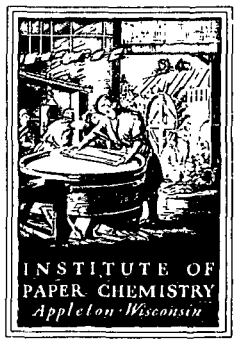

THE INSTITUTE OF PAPER CHEMISTRY
Appleton, Wisconsin 54911
Phone 414/734-9251

September 25, 1972

Project 3020

Dear Dr. Hider:

Here is Progress Report Two for Project 3020. It is questionable whether the additional data obtained during the delay warranted holding up the report since verification of those results is still needed. As usual, any comments on the report that you may have would be welcome.

Sincerely yours,

Morris A. Johnson

Morris A. Johnson
Research Fellow
Division of Natural
Materials & Systems

MAJ/jks
Enclosure
Copies to: Dr. R. B. Valley
Mr. F. A. Adams
Dr. T. R. Santelli

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

UTILIZATION OF PULP MILL EFFLUENT IN THE PRODUCTION OF PAPERMAKING MYCELIA

Project 3020

Report Two

A Progress Report

to

MEMBERS OF GROUP PROJECT 3020

September 20, 1972

MEMBERS OF GROUP PROJECT 3020

Green Bay Packaging, Inc.

The Mead Corporation

Olinkraft, Inc.

Owens-Illinois, Inc.

St. Regis Paper Company

The Weston Paper and Manufacturing Company

TABLE OF CONTENTS

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

UTILIZATION OF PULP MILL EFFLUENT IN THE PRODUCTION OF PAPERMAKING MYCELIA

SUMMARY

Five more fungi were grown on defined media and the mycelia incorporated into handsheets. The paper evaluation data generally show decreases in strength properties with increasing amounts of mycelia incorporation; however, in four of the five cases final judgment of these tests should be reserved pending a small amount of further testing. Effects of variables in handsheet preparation, such as control pulp, beating of mycelia, and freeze-drying of mycelia have been examined. In Phase II work, two fungi have been found to grow rather well on NSSC spent liquor fortified with nitrogen and phosphorus. All detectable acetate and 20 to 40% of the carbohydrate is removed from the spent liquor during log phase growth which should result in a substantial lowering of biochemical oxygen demand.

INTRODUCTION

As projected in the first report, work was begun on Phase II (growth of fungi on spent liquor) of this project in this reporting period to assess the nature and magnitude of the effort that would be necessary to grow the fungi on NSSC spent liquor. Orientation in Phase II has been time consuming with the result that our efforts to carry on Phase I (growth of fungi on defined media with subsequent handsheet evaluation) research simultaneously have been restricted somewhat. Nevertheless, in our own judgment, we have proceeded in a manner that most efficiently approaches the goals of both phases on the limited budget available. Experience gained in this past year is of considerable value in probing this essentially untapped field, particularly in the sense that we are beginning to appreciate what lies within the realm of possibilities in Phases I and II.

At this juncture we have eleven species in culture. At least one species of each of four major cell-wall composition classes has been grown and evaluated in handsheets containing from 5 to 50% mycelia. Seven organisms have been monitored for their ability to grow on NSSC spent liquor although the investigation has been more thorough in two cases than for the entire group.

MATERIALS AND METHODS

FUNGI

In addition to organisms listed in Report One, the following were purchased from the American Type Culture Collection: Daedalea unicolor (ATCC No. 9405), Polyporus anceps (ATCC No. 13242), Schizophyllum commune (ATCC No. 9418, also known as Flammulina velutipes), and Trametes suaveolens (ATCC No. 9417). A culture of Rhizidiomyces sp. was obtained gratis from Dr. Melvin S. Fuller of the University of Georgia.

MEDIA

The four organisms above, which were obtained from the ATCC, grew on the defined asparagine-glucose medium described in Report One; however, since the growth rate was slow (maybe simply a prolonged lag period, since growth did appear to speed up later with the possible exception of P. anceps), they were grown on 1.5% malt extract instead. Rhizidiomyces sp. was cultured in a medium containing the following constituents in grams/liter at pH 7.2: yeast extract 1.0, soluble starch 3.75, K_2HPO_4 0.25, and $MgSO_4$ 0.125 (1).

Phase II media development constitutes part of the experimental program of this report, and various formulations are tabulated with the corresponding results. While a general statement may be foolhardy, it is reasonable to state that, in most cases, dilution of the NSSC spent liquor plus nitrogen and phosphorus supplementation is necessary for growth. No attempt has been made to use procedures like stripping or precipitation in liquor pretreatment.

CULTURE TECHNIQUES

Growth conditions were as previously described. Both the minifermentors and Erlenmeyer shake flasks were employed; the growth container is noted with the data where deemed to be of some relevance. Some variables connected with the processing of harvested mycelia are described under Results.

HANDSHEET PREPARATION AND EVALUATION

Procedures in Report One were followed unless noted otherwise. Some air drying at 50°C. overnight was conducted as noted in the tables. The control pulp was again aspen kraft except that spruce kraft (Oxford) was used in one case where the control pulp was the variable under study.

ANALYSIS OF CULTURE FILTRATES FOR ACETATE, CARBOHYDRATE, AND LIGNIN

While the initial analysis of the NSSC spent liquor was conducted by our analytical department (Tables IX and X), other techniques were devised for routine analysis of the many samples resulting from growth curve studies. For this purpose, acetic acid analysis was conducted by direct gas chromatography of culture filtrates. Twenty microliters of culture filtrate acidified with phosphoric acid to pH 2 were injected into a 6 ft. x 1/8 in. stainless steel column packed with Porapak Q and fitted with a removable 6-in. precolumn of the same type. A glass insert was used in the injector port to accumulate nonvolatiles and replaced periodically. Gas chromatographic analysis was conducted at a column temperature of 170°C., injector at 200°C., thermal conductivity detector at 285°C., and a helium flow rate of 25 cc./min. Quantitation was by measurement of the acetic acid peak areas with a planimeter with reference to a known acetic acid standard. Retention time was 9.3 min.

The disappearance of lignin was followed simply by examination of the ultraviolet spectra of the culture filtrates. Undoubtedly, more than lignin is responsible for the ultraviolet peak at 270 nm.; nevertheless, within limitations discussed later, this simple procedure does provide an estimate of the utilization of ultraviolet-absorbing compounds by a given organism. Any minor liquor component contributing to the absorption would also be monitored, but this is not undesirable. Should an organism be found that readily destroys the ultraviolet peak it would no doubt be of considerable interest and further refinements of this procedure might then be attempted. The peak seen in the liquor is given in Fig. 1.

Sugar analysis was conducted by an indole colorimetric procedure for total carbohydrate (2). A breakdown of the carbohydrate analysis showed the following percentages of various sugars in the liquor: rhamnose, 0.106%; arabinose, 0.111%; xylose, 0.564%; mannose, 0.029%; galactose, 0.180%; glucose, 0.122%. The indole colorimetric peaks for 15 micrograms of each of these sugars and for the mixture encountered in the NSSC spent liquor are shown in Fig. 2. The compromise wavelength chosen for the particular mixture was 470 nm.

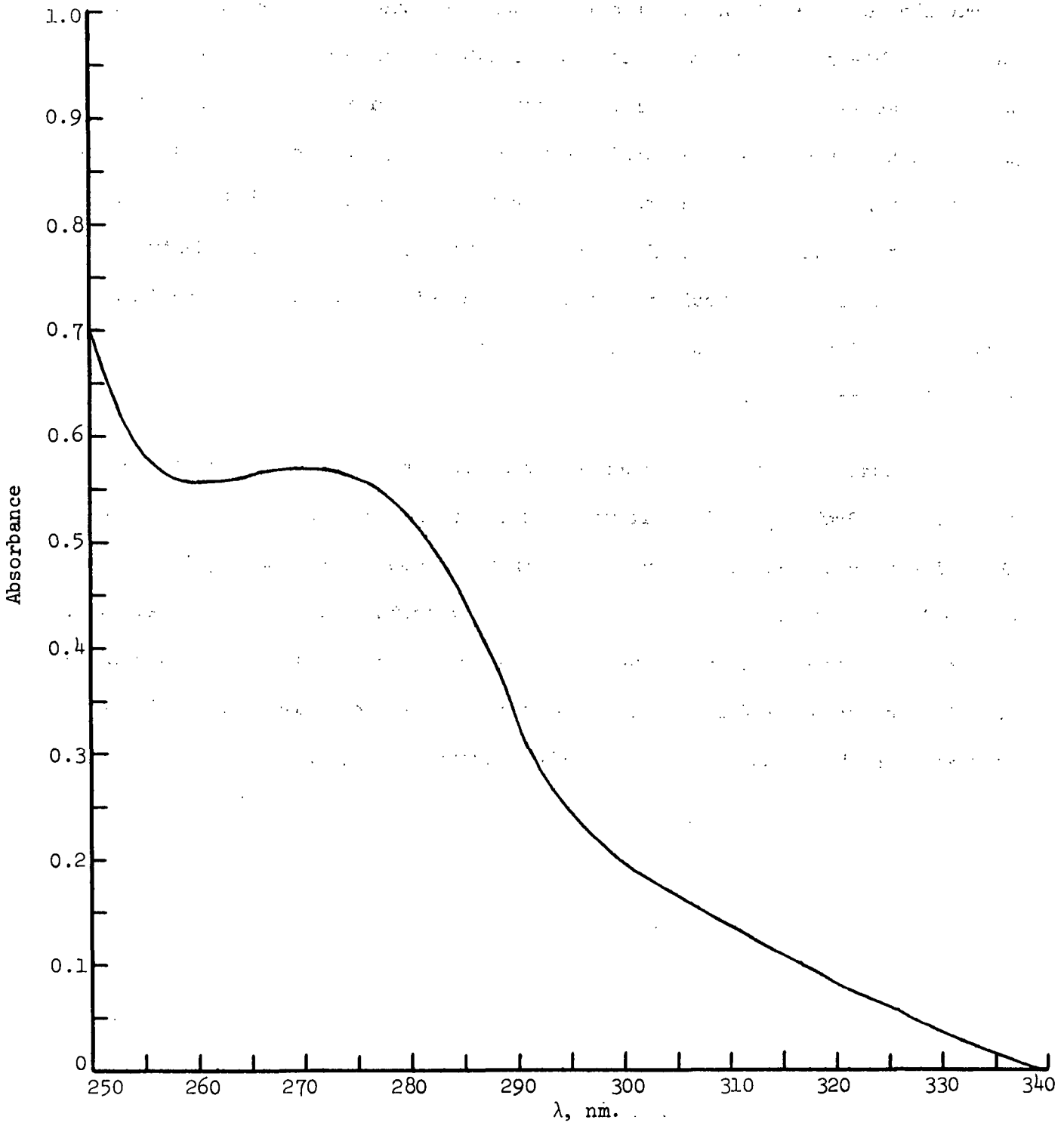

Figure 1. Ultraviolet Spectrum of NSSC Spent Liquor

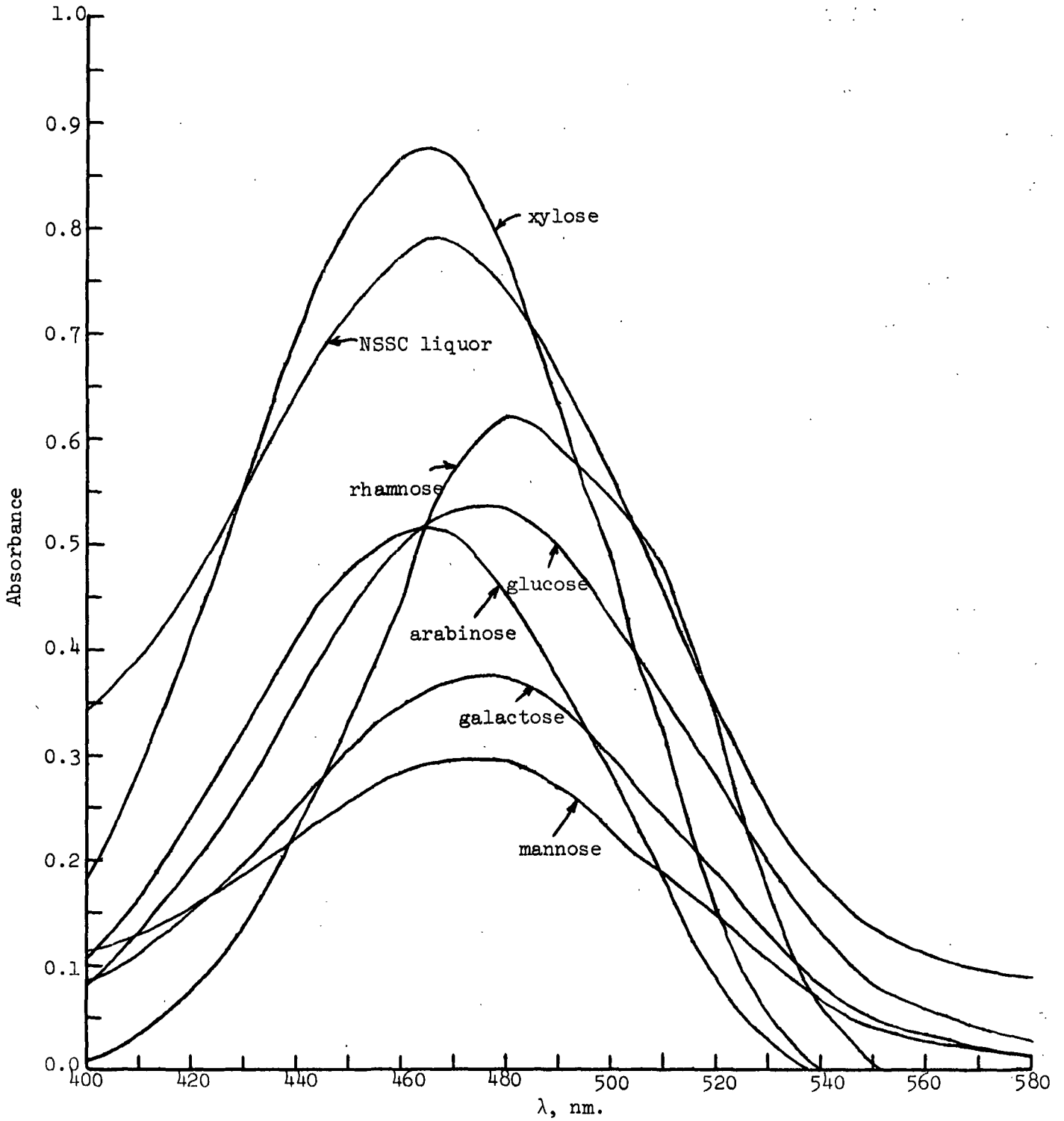

Figure 2. Visible Spectra of Colors Obtained by the Reaction of Indole with Sugar Constituents of NSSC Spent Liquor

RESULTS

PHASE I INVESTIGATIONS

Rhizidiomyces sp., D. unicolor, P. anceps, T. suaveolens, and S. commune were grown and incorporated into handsheets at several levels. The yields and growth rates encountered are presented in Table I. The handsheet evaluation data are given in Table II and in Fig. 3-7. Freeness and drainage results appear in Table III. A brief examination of the growth of a still versus a shaken culture of Saprolegnia ferax is also incorporated in Table I. It can be seen that the shaking rate employed routinely increases the yield of S. ferax about threefold.

TABLE I

GROWTH OF VARIOUS FUNGI

Organism	Culture	Days	Yield, g.	Growth Rate, g./l./day
Rhizidiomyces sp.	Minifermentor	8	0.81	0.17
	Minifermentor	7	0.74	0.15
		7	0.62	0.13
		7	0.19[a]	0.04[a]
	Shake	7	7.47	0.14
		7	7.47	0.14
D. unicolor	Shake[b]	6	1.40	0.07
P. anceps	Shake[b]	6	1.82	0.08
S. commune	Shake[b]	6	1.25	0.06
T. suaveolens	Shake[b]	6	2.49	0.12
S. ferax	Shake	5	4.54	0.76
S. ferax	Still	5	1.52	0.26

[a] No aeration.

[b] Malt extract medium.

TABLE II

MYCELIAL PAPER EVALUATION DATA

Organism	Mycelia, %	Basis Weight, g./m.^{2}, o.d.	Thickness, µm.	Density, g./cc.	Moisture, %	Burst Factor	Bendtsen Porosity, ml./min.	Tensile, km.	Stretch, %	Tear Factor
Rhizidiomyces sp.	0	59.7	83.4	0.716	8.0	45.9	423	9.14	2.0	85.1
	5	59.8	97.0	0.616	8.1	39.5	537	8.20	2.0	78.9
	10	60.4	106.0	0.571	8.3	35.8	644	7.44	1.9	73.5
	20	60.0	112.0	0.536	8.6	27.8	983	5.86	1.8	64.7
	25	60.0	108.0	0.553	8.5	28.8	862	6.39	1.8	70.7
	50	60.7	122.0	0.499	9.6	12.0	3290	2.78	1.5	34.9
P. anceps	0	60.7	85.2	0.712	7.3	51.3	332	9.22	2.26	90.3
	5	60.6	84.8	0.715	7.5	43.0	224	8.10	1.82	81.8
	10	59.5	84.7	0.702	7.6	38.5	137	7.79	1.80	78.7
	20	60.7	86.0	0.706	8.1	32.3	66	6.70	1.42	66.6
	50	60.2	86.6	0.695	9.1	20.0	23	4.82	1.10	41.2
D. unicolor	0	60.7	85.2	0.712	7.3	51.3	332	9.22	2.26	90.3
	5	60.5	83.9	0.721	7.4	41.8	254	7.51	1.64	81.3
	10	60.5	84.4	0.717	7.6	39.7	204	7.17	1.59	77.4
	20	60.0	83.4	0.719	7.9	32.2	107	6.04	1.28	66.7
	50	60.0	83.4	0.719	9.0	14.9	44	4.34	1.08	43.3
T. suaveolens	0	60.7	85.2	0.712	7.3	51.3	332	9.22	2.26	90.3
	20	60.4	84.7	0.713	8.0	38.3	53	6.92	1.64	62.3
	50	58.8	82.3	0.714	9.2	20.9	<12	3.88	0.90	36.1
S. commune	0	60.7	85.2	0.712	7.3	51.3	332	9.22	2.26	90.3
	5	60.9	84.4	0.722	7.4	44.4	250	8.07	1.96	81.4
	10	60.8	84.1	0.723	7.6	41.2	167	6.97	1.50	77.6
	20	60.8	84.1	0.723	7.9	40.0	63	6.67	1.55	63.8
	50	60.2	84.3	0.714	9.0	19.6	<12	4.18	0.98	38.5

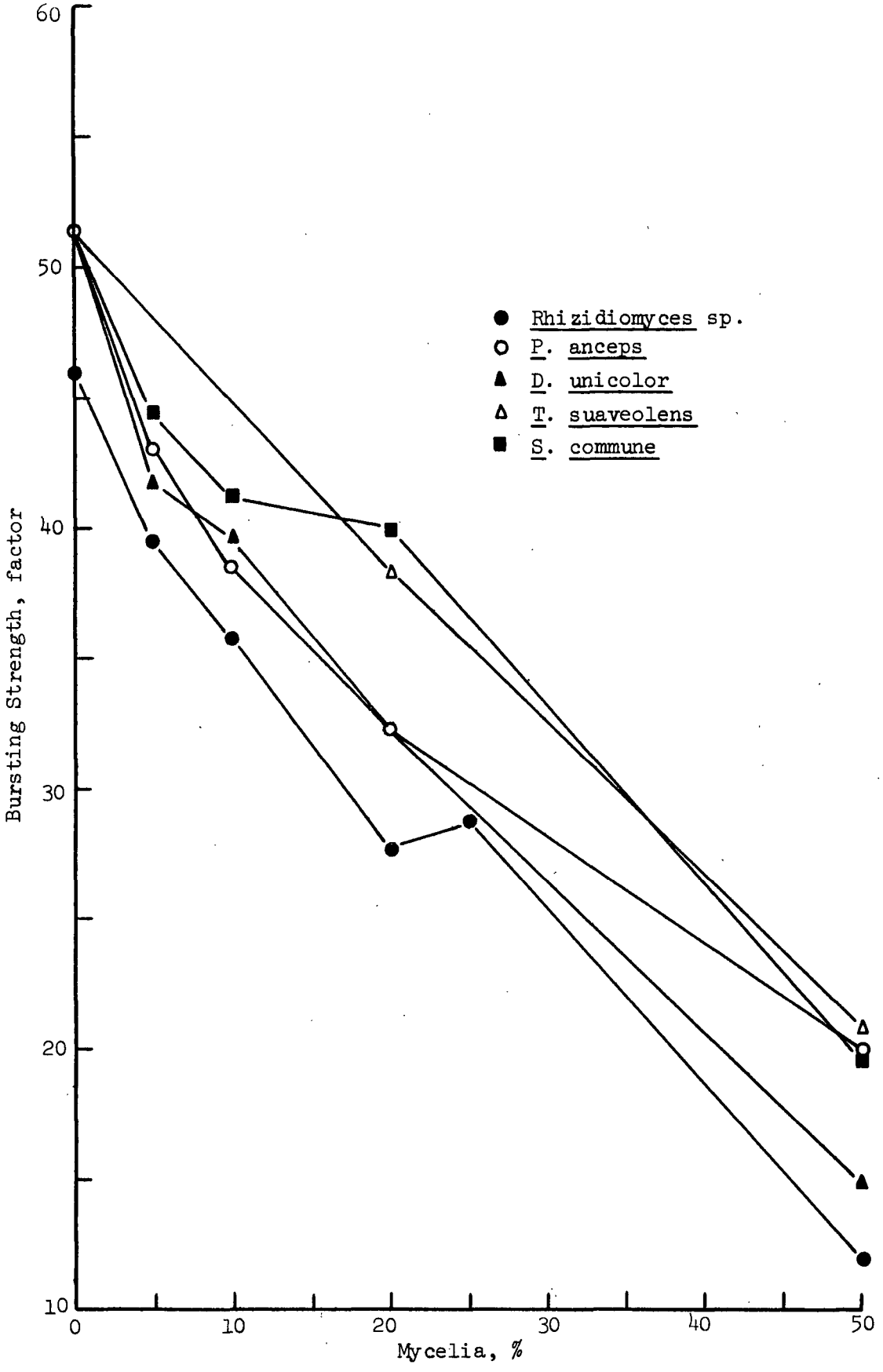

Figure 3. Bursting Strength of Mycelial Paper

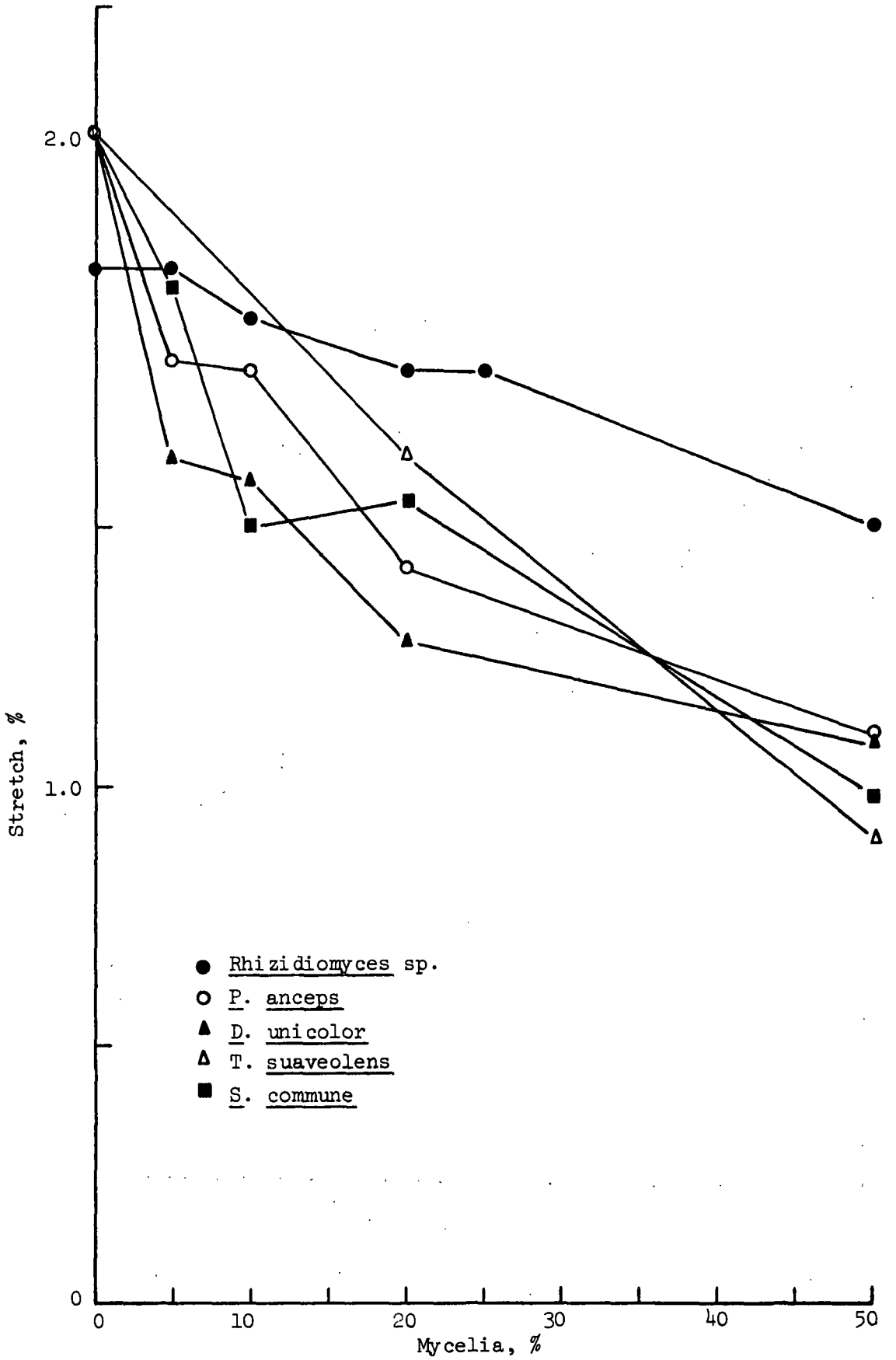

Figure 4. Stretch of Mycelial Paper

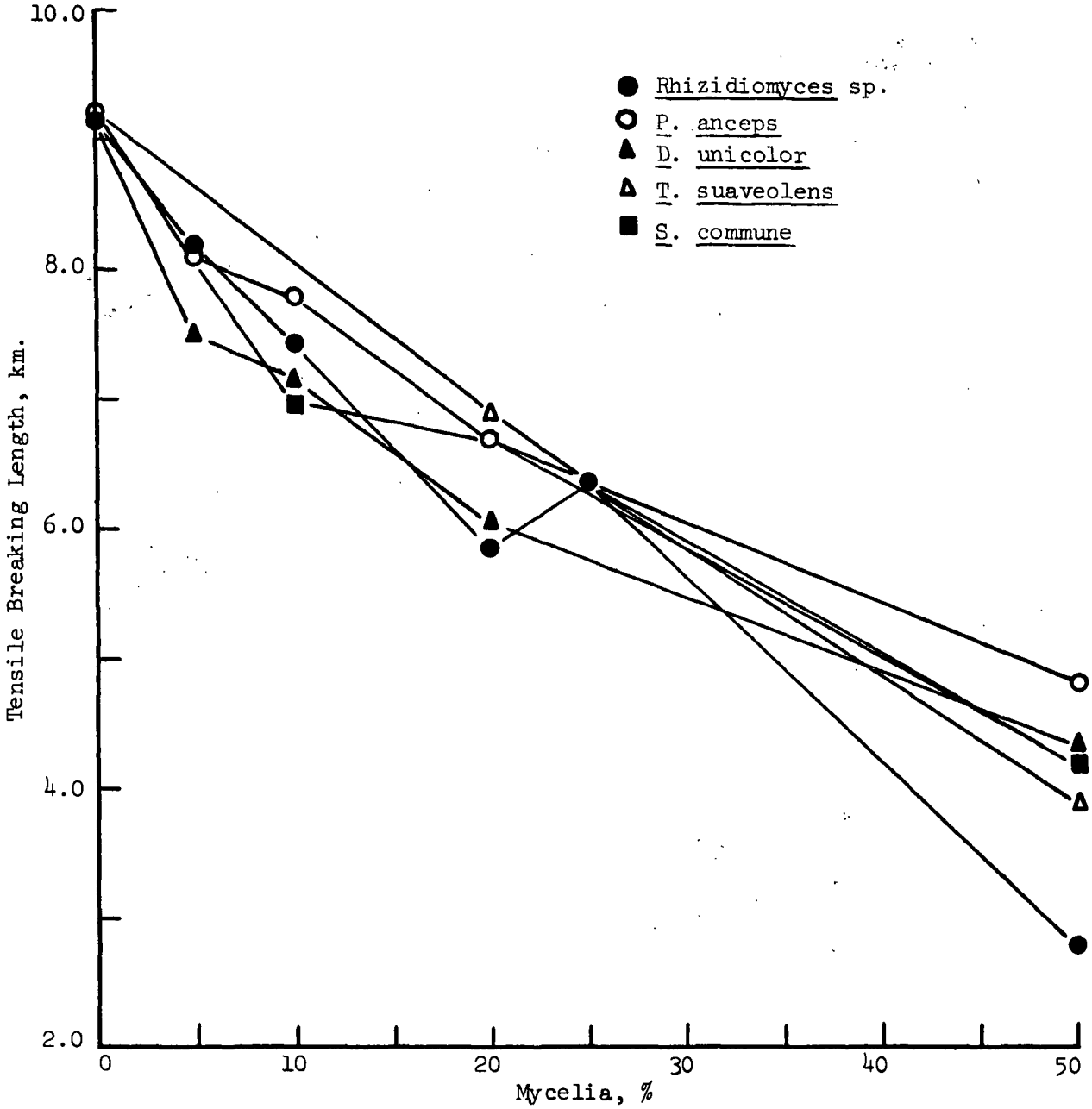

Figure 5. Tensile Strength of Mycelia Paper

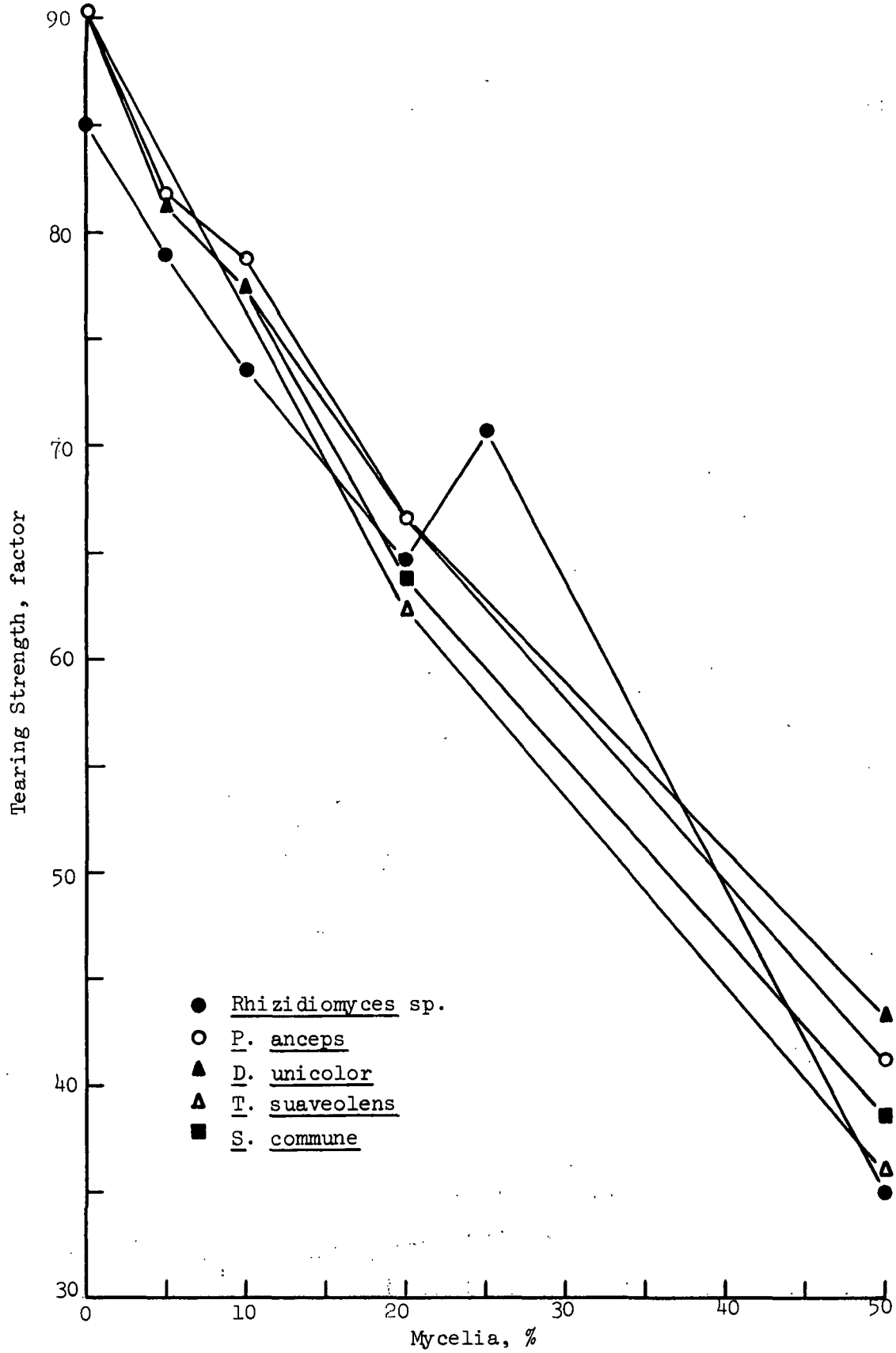

Figure 6. Tearing Strength of Mycelia Paper

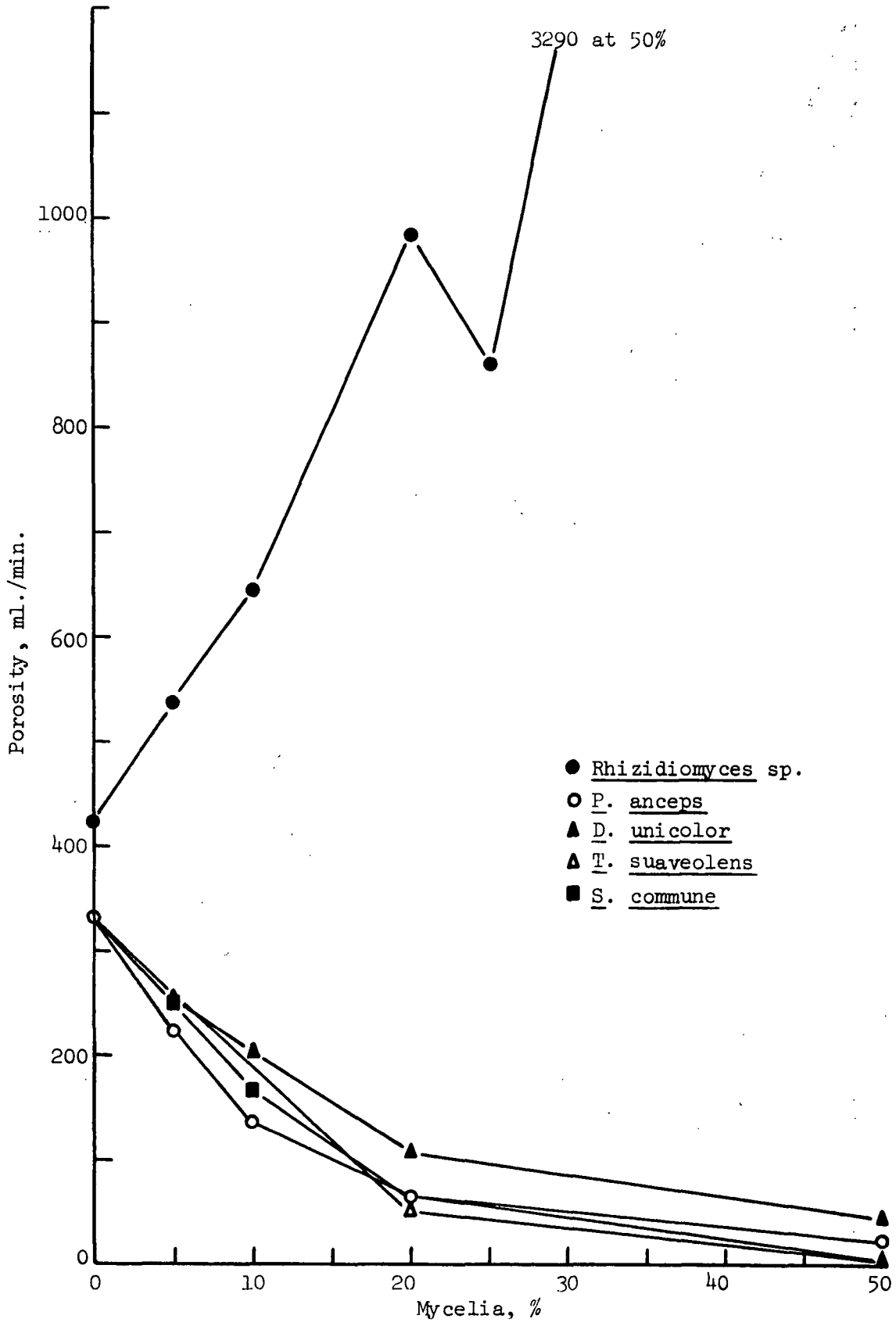

Figure 7. Porosity of Mycelial Paper

TABLE III

FREENESS AND DRAINAGE FOR VARIOUS PULP-MYCELIA MIXTURES

Mycelia, %	Freeness, C.S., ml.					Drainage Time, sec.				
	R.[a]	P. a.	D. u.	T. s.	S. c.	R.	P. a.	D. u.	T. s.	S. c.
0	505	500	500	500	500	5.3	5.2	5.2	5.2	5.2
5	515	480	485	N.D.[b]	490	5.1	5.2	5.2	N.D.[b]	5.4
10	505	425	435	N.D.[b]	400	5.1	5.8	5.5	N.D.[b]	6.1
20	545	370	340	265	315	4.9	7.5	6.6	9.5	8.5
25	560	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	4.9	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]
50	585	175	155	70	100	4.8	22.6	19.3	43.0	28.2

[a] Organism abbreviations.

[b] N.D. = not determined.

The effect of employing a stronger control pulp in the preparation of mycelia-wood pulp mixtures for handsheet formation was investigated using Pythium debaryanum mycelia combined with aspen pulp versus spruce pulp. The handsheet evaluation data for the mycelia-aspen and mycelia-spruce mixtures are compared in Table IV.

It may be recalled from the first report that mycelia-wood pulp mixtures were given an arbitrary 600 counts on the British disintegrator prior to the formation of handsheets. A study of this "beating" time on subsequent behavior of a mycelia-pulp mixture was conducted with a mixture of 20% S. ferax mycelia and 80% aspen kraft pulp. Five 10-gram batches of the 20% mixture were prepared, and a single batch used for each beating time, namely, 50, 300, 600, 1200, and 3000 counts on the British disintegrator. Three 10-gram batches of 100% aspen kraft pulp control were treated identically but only at the 50, 600, and 3000 count levels. A comparison of the freeness and drainage results for the 20% mixture versus control is presented in Table V. The handsheet data appear in Table VI.

More S. ferax was grown to examine the effect of freeze-drying upon subsequent behavior of mycelia-pulp mixtures. The freeness and drainage data for freeze-dried versus never-dried mycelia were obtained as shown in Table VII. Table VIII presents the handsheet evaluation results.

TABLE IV

EFFECT OF CONTROL PULP IN MYCELIA-PULP MIXTURES

P. debaryanum Mycelia, %	Control Pulp	Basis Weight, g./m.^{2}, o.d.	Thickness, µm.	Density, g./cc.	Moisture, %	Burst Factor	Bendtsen Porosity, ml./min.	Tensile, km.	Stretch, %	Tear Factor
0	Aspen	61.0	83.2	0.733	7.9	48.3	285	8.82	2.0	80.7
5		61.4	84.9	0.723	8.1	46.8	236	7.69	2.0	71.7
20		60.5	83.0	0.729	8.4	41.9	77	7.10	2.0	62.8
50		62.5	82.2	0.760	9.2	29.4	<12	4.71	1.3	36.5
0	Spruce	60.2	89.8	0.670	8.0	65.7	243	8.99	2.9	108
5		60.5	90.7	0.667	8.2	65.0	101	8.41	3.2	106
10		59.8	88.4	0.676	8.3	63.9	88	8.07	3.1	96.3
20		60.1	86.2	0.697	8.4	56.7	31	6.92	2.8	83.2
25		59.6	84.8	0.703	8.6	50.2	17	6.53	2.7	73.8
50		60.3	83.6	0.721	9.2	27.4	<12	3.92	1.1	N.D.[a]

[a] N.D. = not determined.

TABLE V

FREENESS AND DRAINAGE OF A MYCELIA-PULP MIXTURE VERSUS BEATING TIME

S. ferax Mycelia, %	British Disintegrator, counts	Freeness, C.S., ml.	Drainage Time, sec.
0	50	505	5.2
	600	500	5.4
	3000	495	5.5
20	50	290	11.4
	300	280	11.9
	600	275	12.0
	1200	250	12.7
	3000	235	15.5

TABLE VI

HANDSHEET EVALUATION DATA OF A MYCELIA-PULP MIXTURE VERSUS BEATING TIME

S. ferax Mycelia, %	British Disintegrator, counts	Basis Weight, g./m.^{2}, o.d.	Thickness, µm.	Density, g./cc.	Moisture, %	Burst Factor	Bendtsen Porosity, ml./min.	Tensile, km.	Stretch, %	Tear Factor
0	50	59.6	84.9	0.702	8.1	44.1	369	8.79	1.9	88.6
	600	61.3	86.6	0.708	8.1	48.6	277	8.89	2.1	90.3
	3000	59.5	83.4	0.713	8.0	49.4	242	9.34	2.2	87.7
20	50	60.5	89.9	0.673	8.9	46.0	39	8.34	1.9	66.1
	300	61.1	89.4	0.683	8.8	47.1	28	8.60	2.0	66.0
	600	61.3	89.4	0.686	8.9	49.2	24	8.98	2.1	65.8
	1200	59.4	86.4	0.688	8.9	46.9	25	9.37	2.1	64.6
	3000	61.5	88.4	0.696	9.0	51.2	19	8.35	2.1	62.4

TABLE VII

FREENESS AND DRAINAGE FOR FREEZE-DRIED
VERSUS NEVER-DRIED S. FERAX

Mycelia, %	Aspen, %	Freeness, C.S., ml.	Drainage Time, sec.
Freeze-dried			
0	100	490	5.3
5	95	415	6.5
10	90	365	8.3
20	80	275	12.2
25	75	240	16.1
50	50	140	34.0
Never-dried			
0	100	505	5.5
5	95	445	6.3
10	90	370	8.2
20	80	245	13.9
25	75	200	18.9
50	50	90	45.0

TABLE VIII

HANDSHEET EVALUATIONS OF MYCELIAL PAPER MADE FROM FREEZE-DRIED VERSUS NEVER-DRIED S. FERAX

Mycelia, %	Aspen, %	Basis Weight, g./m.^{2}, o.d.	Thickness, µm.	Density, g./cc.	Moisture, %	Burst Factor	Bendtsen Porosity, ml./min.	Tensile, km.	Stretch, %	Tear Factor
Freeze-dried										
0	100	60.4	84.5	0.715	8.1	48.2	331	8.87	2.1	82.1
5	95	61.0	84.0	0.726	8.3	48.3	211	9.02	2.3	80.0
10	90	61.9	83.0	0.746	8.3	53.5	108	9.19	2.3	72.4
20	80	60.2	78.1	0.771	8.9	55.6	31	9.36	2.4	63.1
25	75	60.6	77.3	0.784	9.0	51.4	20	9.34	2.3	58.1
50	50	60.0	70.3	0.853	10.0	40.3	<5	8.36	1.8	37.3
Never-dried										
0	100	59.8	82.3	0.727	7.5	51.7	319	8.76	2.1	85.6
5	95	60.5	81.0	0.747	7.6	54.0	111	9.49	2.4	78.7
10	90	61.1	80.8	0.756	7.8	54.2	46	9.46	2.4	70.0
20	80	60.7	77.9	0.779	8.2	57.0	<5	9.70	2.3	57.3
25	75	60.8	77.1	0.789	8.4	57.1	<5	9.77	2.4	53.3
50	50	59.8	71.2	0.840	9.5	42.2	<5	7.50	1.4	32.1

PHASE II INVESTIGATIONS

NSSC spent liquor was obtained from Green Bay Packaging Incorporated. As received it had a pH of 5.9. Since it contained a small amount of settleable solids, some of it was centrifuged (10,000 x g for 15 minutes) and the supernatant filtered through Whatman No. 1 paper (done because decanting was not a clean operation). An analysis of the liquor was conducted then on both the as-received liquor and the clarified (centrifuged and filtered) supernatant. The two analytical reports appear in Tables IX and X.

For an initial test, dilutions of as-received NSSC spent liquor with distilled water ranging from 1 to 50% liquor were autoclaved and inoculated with our most finicky organism to date, S. ferax. After nine days on the shaker at 29°C. no growth had occurred. Autoclaved as-received NSSC spent liquor was mixed then in low proportions with our standard asparagine-glucose medium (supplemented with yeast extract for S. ferax) and attempts were made to grow several organisms in it. While this was essentially a toxicity study, growth enhancement was observed in some cases as seen in Table XI. A study of the ability of organisms other than S. ferax to grow on various dilutions of as-received NSSC spent liquors with distilled water suggested that rather concentrated liquor could be used to support some growth of several organisms without supplementation. These results are shown in Table XII. As a check on the contribution to the dry weights made by the settleable solids in the as-received NSSC spent liquor, the data of Table XIII for liquor not inoculated and inoculated with M. rouxii was obtained. In other experiments, the effect of asparagine supplementation with M. rouxii was studied; these results also appear in Table XIII.

TABLE IX

ANALYSIS OF NSSC SPENT LIQUOR

		As-Received Basis	Ovendry Solids Basis
Copper content, %		0.000086	0.00062
Iron content, %		0.0030	0.022
Zinc content, %		0.00023	0.0017
Mercury content, µg./l.		None[a]	
%		0.0000004	0.000003
Total carbohydrate, %		1.29	9.32
Total solids, %	Test 1	13.83	
	Test 2	13.85	
	Av.	13.84	
Ash, %	Test 1	8.63	
	Test 2	8.63	
	Av.	8.63	62.36
Phosphorus, %		None[b]	None[c]
Magnesium, %	Test 1	0.0063	
	Test 2	0.0073	
	Av.	0.0068	0.049
Manganese, %	Test 1	0.0047	
	Test 2	0.0047	
	Av.	0.0047	0.034
Silver, %		None[d]	None[e]
Lead, %		None[f]	
Calcium, %	Test 1	0.041	
	Test 2	0.035	
	Av.	0.038	0.27
Molybdenum, %		None[g]	0.02
Total sulfur, %	Test 1	0.83	
	Test 2	0.84	
	Av.	0.84	6.07

See end of table for footnotes.

TABLE IX (Continued)

ANALYSIS OF NSSC SPENT LIQUOR

		As-Received Basis	Ovendry Solids Basis
Acetate, %	Test 1	2.23	
	Test 2	2.22	
	Av.	2.22	16.04
Sodium, %		1.6	12
Kjeldahl nitrogen, mg./l.		<0.8	
Acid-soluble lignin, %	Test 1	4.60	
	Test 2	4.69	
	Av.	4.64	33.5
Acid-insoluble lignin, %	Test 1	0.69	
	Test 2	0.60	
	Av.	0.64	4.62

Note: Unless otherwise indicated, all values are single determinations.

---

[a]Detection limit = 4.
[b]Detection limit = 0.04.
[c]Detection limit = 0.3.
[d]Detection limit = 0.0002
[e]Detection limit = 0.0001
[f]Detection limit = 0.0005
[g]Detection limit = 0.003.

Methods: Copper, iron, and zinc — Atomic absorption spectrophotometry, acid digestion of sample.
Mercury — Flameless atomic absorption spectrophotometry, aqua regia method.
Total carbohydrate — Tappi 53, no. 2:257-61(Feb., 1970).
Total solids — Dried at 105°C. for 24 hours.
Ash — Ashed at 650°C. for 1 hour.
Metals — Emission spectroscopy.
Total sulfur — Marathon Research Method.
Acetate — TAPPI Standard T 629 os-53.
Sodium — Flame photometry.
Kjeldahl nitrogen — Standard methods of the analysis of water and wastewater, 13th ed., p. 469, 1971.
Acid-insoluble lignin — TAPPI Standard T 13 m-54.

TABLE X

ANALYSIS OF NSSC SPENT LIQUOR —
CENTRIFUGED AND FILTERED

		As-Received Basis	Ovendry Solids Basis
Copper content, %		0.000032	0.00025
Iron content, %		0.0025	0.019
Zinc content, %		0.00026	0.0020
Mercury content, µg./l.		None[a]	
%		0.0000004	0.000003
Total carbohydrate, %		1.11	8.56
Total solids, %	Test 1	12.94	
	Test 2	12.97	
	Av.	12.96	
Ash, %	Test 1	8.16	
	Test 2	8.27	
	Av.	8.22	63.43
Phosphorus, %		None[b]	None[c]
Magnesium, %	Test 1	0.0070	
	Test 2	0.0071	
	Av.	0.0070	0.050
Manganese, %	Test 1	0.0036	
	Test 2	0.0040	
	Av.	0.0038	0.029
Silver, %		None[d]	None[e]
Lead, %		None[f]	
Calcium, %	Test 1	0.019	
	Test 2	0.021	
	Av.	0.020	0.14
Molybdenum, %		None[g]	0.02
Total sulfur, %	Test 1	0.74	
	Test 2	0.77	
	Av.	0.76	5.86

See end of table for footnotes.

TABLE X (Continued)

ANALYSIS OF NSSC SPENT LIQUOR —
CENTRIFUGED AND FILTERED

		As-Received Basis	Ovendry Solids Basis
Acetate, %	Test 1	2.20	
	Test 2	2.20	
	Av.	2.20	16.98
Sodium, %		1.5	12
Kjeldahl nitrogen, mg./l.		<0.6	
Acid-soluble lignin, %	Test 1	4.53	
	Test 2	4.61	
	Av.	4.57	35.3
Acid-insoluble lignin, %	Test 1	0.02	
	Test 2	0.02	
	Av.	0.02	0.15

Note: Unless otherwise indicated, all values are single determinations.

---

[a]Detection limit = 4.
[b]Detection limit = 0.04.
[c]Detection limit = 0.3.
[d]Detection limit = 0.00002.
[e]Detection limit = 0.0002
[f]Detection limit = 0.0005.
[g]Detection limit = 0.003.

Methods: Copper, iron, and zinc — Atomic absorption spectrophotometry, acid digestion of sample.
Mercury — Flameless atomic absorption spectrophotometry, aqua regia method.
Total carbohydrate — Tappi 53, no. 2:257-61(Feb., 1970).
Total solids — Dried at 105°C. for 24 hours.
Ash — Ashed at 650°C. for 1 hour.
Metals — Emission spectroscopy.
Total sulfur — Marathon Research Method.
Acetate — TAPPI Standard T 629 os-53.
Sodium — Flame photometry.
Kjeldahl nitrogen — Standard methods of the analysis of water and wastewater, 13th ed., p. 469, 1971.
Acid-insoluble lignin — TAPPI Standard T 13 m-54.

TABLE XI

MYCELIAL GROWTH IN THE PRESENCE OF DILUTE NSSC LIQUOR

Organism	NSSC Liquor, %[b]	Incubation, days	Filtrate pH	Freeze-Dry Weight, mg.
S. ferax	0	11	7.46	330
	1	11	6.94	450
	5	11	7.65	240
	10	11	5.93	0
P. debaryanum	0	7	5.50	115
	1	7	4.97	184
	5	7	5.11	169
	10	7	5.85	N.D.[a]
P. parasitica	0	7	4.80	315
	1	7	6.14	N.D.[a]
	5	7	7.54	266
	10	7	5.74	180
Fusarium sp.	0	7	6.49	119
	1	7	6.50	150
	5	7	7.66	183
	10	7	8.46	173
M. rouxii	0	7	5.91	140
	1	7	6.57	156
	5	7	7.22	215
	10	7	8.63	204

[a] N.D. = not determined.

[b] See text for media detail.

TABLE XII

MYCELIAL GROWTH ON NSSC LIQUOR WITHOUT SUPPLEMENTATION

Organism	NSSC Liquor, %	Incubation, days	Initial pH	Filtrate pH	Air-Dry[b] Weight, mg.
P. debaryanum	1	7	6.05	8.38	13
	3	7	6.05	8.50	19
	5	7	6.04	8.46	25
	10	7	6.01	5.88	22
	20	7	5.98	5.80	47
	50	7	5.95	N.D.[a]	N.D.[a]
P. parasitica	1	7	6.05	8.43	17
	3	7	6.05	8.78	27
	5	7	6.04	8.72	36
	10	7	6.01	7.65	42
	20	7	5.98	5.80	49
	50	7	5.95	N.D.[a]	N.D.[a]
Fusarium sp.	1	7	6.05	8.40	6
	3	7	6.05	8.73	14
	5	7	6.04	8.48	23
	10	7	6.01	8.47	47
	20	7	5.98	8.20	77
	50	7	5.95	6.99	182
M. rouxii	1	7	6.05	8.21	4
	3	7	6.05	8.65	14
	5	7	6.04	8.82	22
	10	7	6.01	8.75	42
	20	7	5.98	8.57	76
	50	7	5.95	8.48	N.D.[a]
P. cinnamomi	1	7	6.05	8.40	9
	3	7	6.05	8.71	22
	5	7	6.04	8.61	28
	10	7	6.01	8.55	53
	20	7	5.98	8.53	88
	50	7	5.95	N.D.[a]	N.D.[a]

[a] N.D. = not determined.

[b] Overnight at 50°C.

TABLE XIII

NET GROWTH OF M. ROUXII ON NSSC SPENT LIQUOR;
EFFECT OF ASPARAGINE SUPPLEMENTATION

NSSC Liquor, %	Incubation, days	Asparagine, %	Filtrate pH		Airdry Weight, mg.		
			Inoculated	Uninoculated	Inoculated	Uninoculated	Net
1	7	0.00	8.32	6.50	9	4	5
3	7	0.00	8.60	6.95	18	8	10
5	7	0.00	8.58	6.83	24	11	13
3	4	0.00	8.44	N.D.[a]	9	8[b]	1
3	4	0.05	8.83	N.D.[a]	16	8[b]	8
3	4	0.10	8.83	N.D.[a]	14	8[b]	6
3	4	0.20	8.86	N.D.[a]	13	8[b]	5
50 clarified	14	0.00	7.32	5.9	37	0	37
50 clarified	14	0.05	9.05	N.D.[a]	115	0[b]	115
50 clarified	14	0.10	8.90	N.D.[a]	140	0[b]	140
50 clarified	14	0.20	8.80	N.D.[a]	164	0[b]	164

[a] N.D. = not determined.

[b] Values not determined directly but correct if no change in control upon incubation in the absence of inocula.

It was apparent that there would be operational advantages in the utilization of centrifuged and filtered NSSC spent liquor and that pH needed examination with regard to the role it played in the growth data. Therefore, a large batch of NSSC spent liquor was centrifuged, the supernatant filtered, and the resulting filtrate autoclaved. Autoclaved filtrate was mixed 1:1 (i.e., 50% NSSC) with autoclaved 0.1M potassium phosphate buffer of pH's 6, 7, and 8; the resulting 900-ml. portions of buffered NSSC liquor had pH's of 6.0, 6.6, and 7.0, respectively. At each of these three pH levels, three 50-ml. portions were removed to three 250-ml. shake flasks (controls). The 750 ml. remaining at each pH level was inoculated with 5 ml. of M. rouxii culture followed by removal of 50-ml. aliquots into fifteen 250-ml. shake flasks. The dry weight yields and the pH's of filtrates after the recovery of mycelia are given in Fig. 8. Nearly identical results were obtained for NSSC liquor which was not clarified before use (note that the value of interest in this regard is the difference between the weights of inoculated sample and uninoculated control). A single attempt to study the growth of M. rouxii in a similar fashion below pH 6.0 failed; this work needs repetition. Paradoxically (perhaps) the best yields in Fig. 8 occurred where the initial pH was lowest and the final pH was highest. These pH changes will be discussed later. A positive effect (not shown) of nitrogen (asparagine) supplementation in the presence of phosphate buffer was quite evident in these results at 14 days where the initial pH was 6.0.

Heavy metals did not appear to be present in the NSSC spent liquor in inhibitory quantities; nevertheless, a study of M. rouxii growth on the clarified liquor as affected by the presence of a chelator (EDTA) was conducted. No indication of growth inhibition release by EDTA is evident in Table XIV where the NSSC concentration was 50% at an initial pH of 6.0 in 0.05M phosphate buffer.

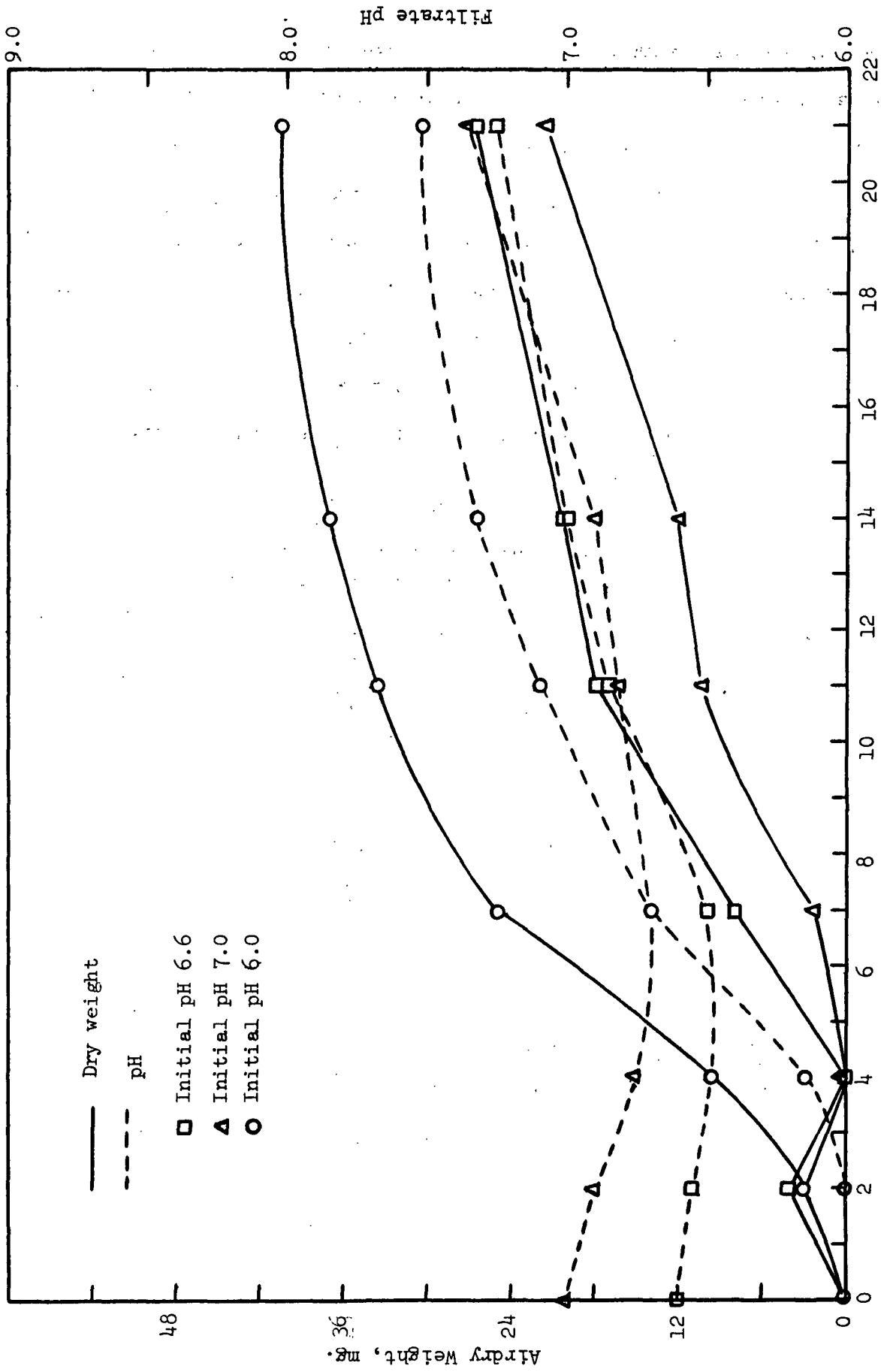

Figure 8. Yields and pH Changes During Buffered Growth of M. rouxii

Table XV shows that actually M. rouxii grows at about the same rate in buffered as in unbuffered NSSC spent liquor and regardless of whether the NSSC spent liquor has been clarified (no nitrogen supplement). When one compares nitrogen and phosphorus-supplemented M. rouxii growth on glucose versus NSSC spent liquor (20%), it is found that the spent liquor does contain a good carbon source for the organism and also can supply the needed trace elements for growth (Table XVI). It should be noted that the addition of 20% NSSC is approximately equivalent to the addition of only 0.2% wood sugars; furthermore, growth on glucose results in an opposite change in filtrate pH from growth on the spent liquor.

TABLE XIV

M. ROUXII GROWTH ON 50% NSSC SPENT LIQUOR IN THE PRESENCE OF EDTA

Na_2 EDTA, M	Asparagine, %	Filtrate pH	Airdry Weight, mg.
0	0.00	6.00	7
10^{-5}	0.00	6.00	7
10^{-4}	0.00	6.05	7
10^{-3}	0.00	6.15	10
10^{-2}	0.00	5.80	0
0	0.05	6.35	26
10^{-5}	0.05	6.15	16
10^{-4}	0.05	6.40	30
10^{-3}	0.05	6.40	26
10^{-2}	0.05	5.75	1

TABLE XV

SIX-DAY GROWTH OF M. ROUXII WITHOUT NITROGEN SUPPLEMENTATION ON NSSC SPENT LIQUOR: EFFECT OF LIQUOR PRETREATMENT AND PHOSPHATE BUFFERING AT pH 6.0

NSSC Liquor, %		pH 6.0 Potassium Phosphate, M	Filtrate pH		Air-Dry Weight		Net
			Sample	Control[a]	Sample	Control[a]	
As received	1	0	8.05	6.00	13	6	7
	3	0	8.15	6.00	22	13	9
	5	0	8.20	6.00	32	21	11
	10	0	8.10	5.95	55	39	16
	20	0	7.95	5.90	106	90	16
	50	0	7.05	5.90	310	295	15
Clarified	1	0	8.10	5.90	9	0	9
	3	0	8.15	5.95	11	0	11
	5	0	8.15	5.90	12	0	12
	10	0	8.05	5.90	15	0	15
	20	0	7.80	5.90	20	0	20
	50	0	6.75	5.90	21	0	21
Clarified	1	0.002	6.10	6.10	10	0	10
	3	0.006	6.10	6.10	10	0	10
	5	0.010	6.20	6.05	13	0	13
	10	0.020	6.20	6.00	12	0	12
	20	0.040	6.20	5.95	20	0	20
	50	0.100	5.90	5.85	8	0	8

[a]No inoculum.

$(NH_4)_2HPO_4$ is a compound which conveniently provides nitrogen and phosphorus supplementation with a single addition. Effects on M. rouxii growth of providing various levels of $(NH_4)_2HPO_4$ in combination with several NSSC spent liquor concentrations are presented in Table XVII. A similar six-day study including 100% spent liquor as the base medium is shown in Fig. 9. Presented in Table XVIII are data which allow comparison of $(NH_4)_2HPO_4$ supplemented growth on the NSSC spent liquor (Fig. 9) with growth on weak spent liquor (used to provide trace elements) to which carbon sources known to be present* in the

*Hydroquinone was used to model lignin.

liquor were added. An attempt to refine the % NSSC spent liquor optimum for M. rouxii at constant $(NH_4)_2HPO_4$ supplementation is depicted in Table XIX. A similar study with more organisms, portrayed in Fig. 10, leads to the conclusion that an optimum lies near 50% NSSC spent liquor for M. rouxii and Fusarium sp. Several other organisms were also tried but responded about like P. parasitica. Lack of growth by the other organisms is in conflict with the results of Table XII; reasons for this are not yet ascertained.

TABLE XVI

FOUR-DAY GROWTH OF M. ROUXII ON NITROGEN AND PHOSPHORUS SUPPLEMENTED NSSC SPENT LIQUOR VERSUS GLUCOSE

Glucose, %	NSSC Liquor, %	KH_2PO_4, %	$(NH_4)_2HPO_4$, %	Trace Elements	pH		Airdry Weight, mg.
					Initial	Filtrate	
2	0	0.1	0.4	+	7.2	2.3	104
4	0	0.1	0.4	+	7.2	2.3	113
2	0	0.0	0.4	+	7.8	2.3	99
2	0	0.0	0.4	-	7.9	3.6	24
2	20	0.1	0.4	+	6.9	5.0	113
0	20	0.1	0.4	+	6.9	8.1	111
0	20	0.0	0.4	-	7.3	8.1	107
0	20	0.1	0.4	-	7.0	8.1	106

With the foregoing information at hand, a partial growth curve showing concomittant filtrate pH values for Fusarium sp. on 50% NSSC spent liquor supplemented with 0.2% $(NH_4)_2HPO_4$ was obtained (Fig. 11). Finally, growth curves, again with concomittant filtrate pH values, were obtained for M. rouxii (Fig. 12) and Fusarium sp. (Fig. 13) at 25% NSSC spent liquor. In both cases, aliquots were removed from the culture filtrates at each point on the growth curves and

TABLE XVII

SIX-DAY GROWTH OF M. ROUXII ON $(NH_4)_2HPO_4$ SUPPLEMENTED NSSC SPENT LIQUOR

NSSC Liquor, %	$(NH_4)_2HPO_4$[a], %	Filtrate pH	Air-Dry Weight, mg.	Air-Dry Weight, net mg.	Efficiency[c]
0	0.0	6.20	6[b]	--	--
0	0.2	6.15	6[b]	--	--
0	0.4	6.15	5[b]	--	--
0	0.8	6.10	6[b]	--	--
1	0.0	8.15	7	1	1.0
1	0.2	6.65	11	5	5.0
1	0.4	6.40	10	5	5.0
1	0.8	6.25	10	4	4.0
3	0.0	8.40	10	4	1.3
3	0.2	7.35	18	12	4.0
3	0.4	6.85	20	15	5.0
3	0.8	6.50	18	12	4.0
5	0.0	8.40	12	6	1.2
5	0.2	8.10	23	17	3.4
5	0.4	7.20	26	21	4.2
5	0.8	6.75	28	22	4.4
10	0.0	8.25	15	9	0.9
10	0.2	8.70	40	34	3.4
10	0.4	8.15	39	34	3.4
10	0.8	7.15	57	51	5.1
20	0.0	8.15	21	15	0.8
20	0.2	8.85	82	76	3.8
20	0.4	8.65	82	77	3.8
20	0.8	8.15	84	78	3.9
50	0.0	7.70	31	25	0.5
50	0.2	8.80	159	153	3.1
50	0.4	8.50	147	142	2.8
50	0.8	7.90	145	139	2.8

[a] Adjusted to pH 6.0

[b] Mostly inoculum weight.

[c] Net mg./% NSSC (may be misleading since growth curve not available at each NSSC level).

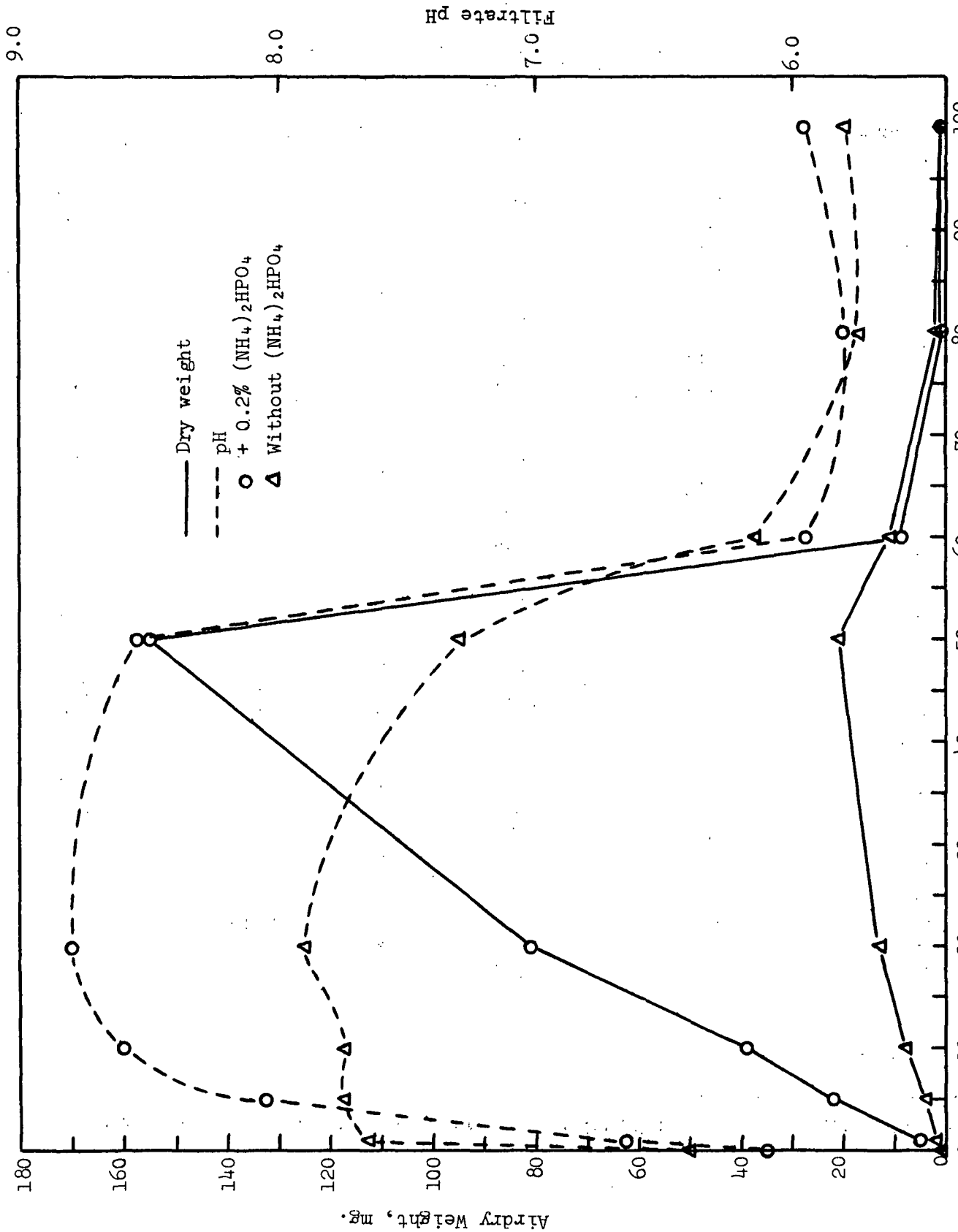

Figure 9. Growth Responses of M. rouxii to NSSC Spent Liquor Concentration and $(NH_4)_2HPO_4$ Supplementation

TABLE XVIII

SIX-DAY MYCELIAL GROWTH ON KNOWN NSSC SPENT LIQUOR CONSTITUENTS OR MODEL COMPOUNDS THEREOF

[Supplemented with 0.2% $(NH_4)_2HPO_4$]

Organism	NSSC Liquor, %	Substrate, %	Filtrate pH[a]	Air-Dry Weight,[a] mg.
		Acetate		
M. rouxii	0	0.00	6.10	0
	0	0.02	6.50	3
	0	1.00	7.20	8
	1	0.00	6.65	5
	1	0.02	6.85	7
	1	1.00	9.10	42
Fusarium sp.	1	0.00	6.85	4
	1	1.00	8.80	70
S. ferax	1	0.00	6.40	0
	1	1.00	6.55	0
P. debaryanum	1	0.00	6.90	4
	1	1.00	6.50	0
Rhizidiomyces sp.	1	0.00	6.90	4
	1	1.00	6.70	2
P. cinnamomi	1	0.00	6.60	3
	1	1.00	6.30	1
P. parasitica	1	0.00	6.65	4
	1	1.00	6.30	1
		Xylose		
M. rouxii	0	0.00	6.10	0
	0	0.02	6.20	4
	0	1.00	6.05	7
	1	0.00	6.65	5
	1	0.02	6.65	8
	1	1.00	3.20	81
Fusarium sp.	1	0.00	6.85	4
	1	1.00	2.90	141
S. ferax	1	0.00	6.40	0
	1	1.00	6.40	1

See end of table for footnote.

TABLE XVIII (Continued)

SIX-DAY MYCELIAL GROWTH ON KNOWN NSSC SPENT LIQUOR CONSTITUENTS OR MODEL COMPOUNDS THEREOF

[Supplemented with 0.2% $(NH_4)_2HPO_4$]

Organism	NSSC Liquor, %	Substrate, %	Filtrate pH[a]	Air-Dry Weight,[a] mg.
P. debaryanum	1	0.00	6.90	4
	1	1.00	6.40	0
Rhizidiomyces sp.	1	0.00	6.90	4
	1	1.00	6.75	10
P. cinnamomi	1	0.00	6.60	3
	1	1.00	6.15	8
P. parasitica	1	0.00	6.65	4
	1	1.00	5.80	16
		Arabinose		
M. rouxii	0	0.00	6.10	0
	0	0.02	6.25	4
	0	1.00	6.15	8
	1	0.00	6.65	5
	1	0.02	6.70	9
	1	1.00	6.10	53
Fusarium sp.	1	0.00	6.85	4
	1	1.00	3.00	118
S. ferax	1	0.00	6.40	0
	1	1.00	6.40	0
P. debaryanum	1	0.00	6.90	4
	1	1.00	6.85	2
Rhizidiomyces sp.	1	0.00	6.90	4
	1	1.00	6.50	28
P. cinnamomi	1	0.00	6.60	3
	1	1.00	6.40	10
P. parasitica	1	0.00	6.65	4
	1	1.00	6.40	6

See end of table for footnote.

TABLE XVIII (Continued)

SIX-DAY MYCELIAL GROWTH ON KNOWN NSSC SPENT LIQUOR CONSTITUENTS OR MODEL COMPOUNDS THEREOF

[Supplemented with 0.2% $(NH_4)_2HPO_4$]

Organisms	NSSC Liquor, %	Substrate, %	Filtrate pH[a]	Air-Dry Weight,[a] mg.
		Hydroquinone		
M. rouxii	0	0.00	6.10	0
	0	0.02	6.30	0
	0	1.00	6.10	1
	1	0.00	6.65	5
	1	0.02	6.50	5
	1	1.00	5.90	0
Fusarium sp.	1	0.00	6.85	4
	1	1.00	6.00	0
S. ferax	1	0.00	6.40	0
	1	1.00	6.00	1
P. debaryanum	1	0.00	6.90	4
	1	1.00	6.00	0
Rhizidiomyces sp.	1	0.00	6.90	4
	1	1.00	6.00	0
P. cinnamomi	1	0.00	6.60	3
	1	1.00	5.60	0
P. parasitica	1	0.00	6.65	4
	1	1.00	5.65	0

[a] Averages of duplicates except for M. rouxii.

analyzed for acetate, carbohydrates, and ultraviolet absorbing materials (primarily lignin) to ascertain what carbon source components of the liquor were utilized for growth. These results appear in Fig. 14 (M. rouxii) and 15 (Fusarium sp.). The data presented in Fig. 11-15 are averages of duplicates except at the very short and very long incubation times.

TABLE XIX

DETERMINATION OF % NSSC SPENT LIQUOR OPTIMUM
AT 0.2% $(NH_4)_2HPO_4$, pH 6.0

(M. rouxii, 7 days incubation)

NSSC Liquor, %	Filtrate pH	Airdry Weight, mg.	Efficiency, mg./% NSSC
20	8.85	74	3.7
25	9.00	98	3.9
30	9.10	114	3.8
35	8.75	137	3.9
40	8.75	146	3.6
45	8.65	162	3.6
50	8.15	94	1.9
55	5.80	4	<0.1
60	5.75	2	<0.1
65	5.75	1	<0.1
70	5.75	1	<0.1

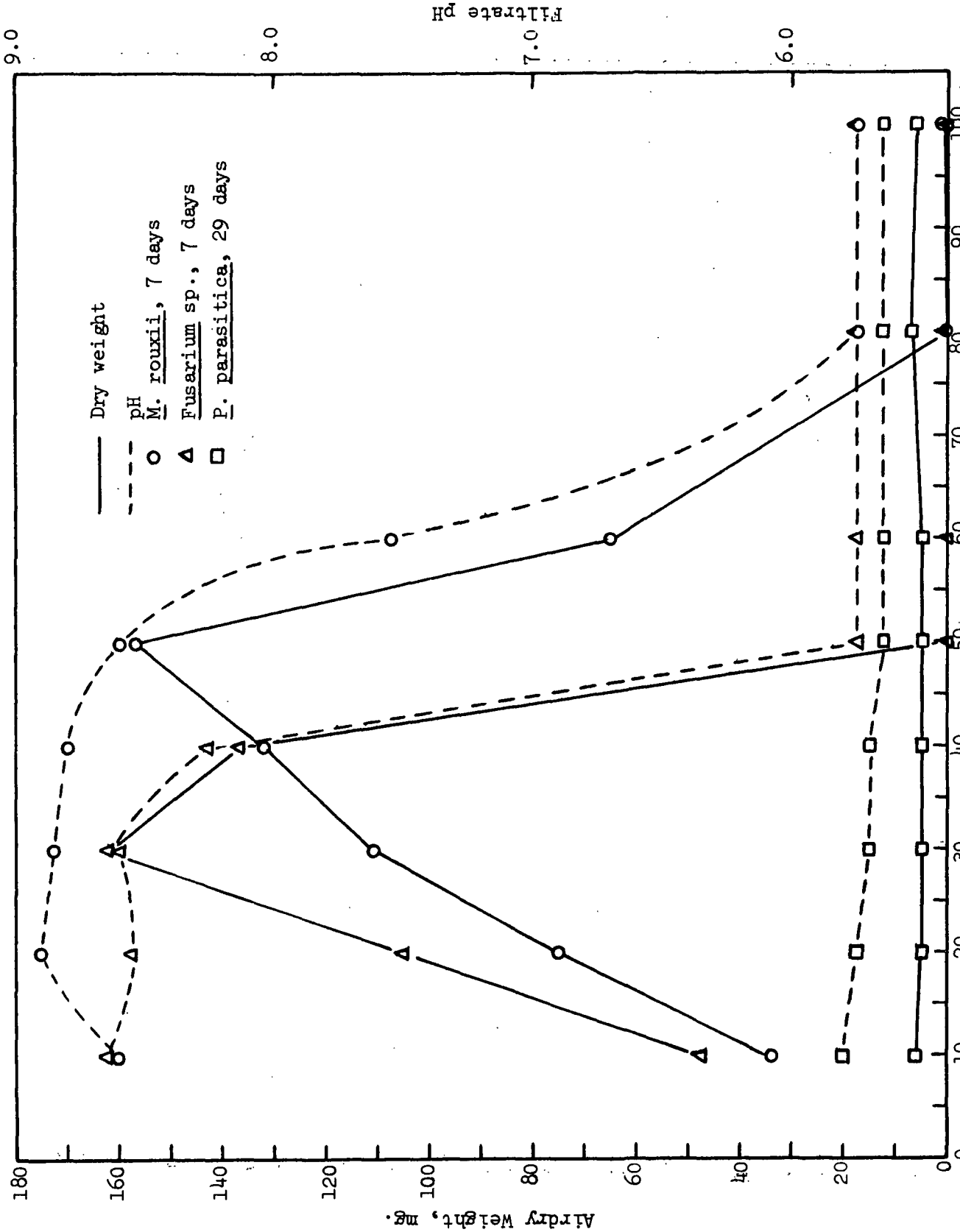

Figure 10. NSSC Spent Liquor Optima at 0.2% $(NH_4)_2HPO_4$

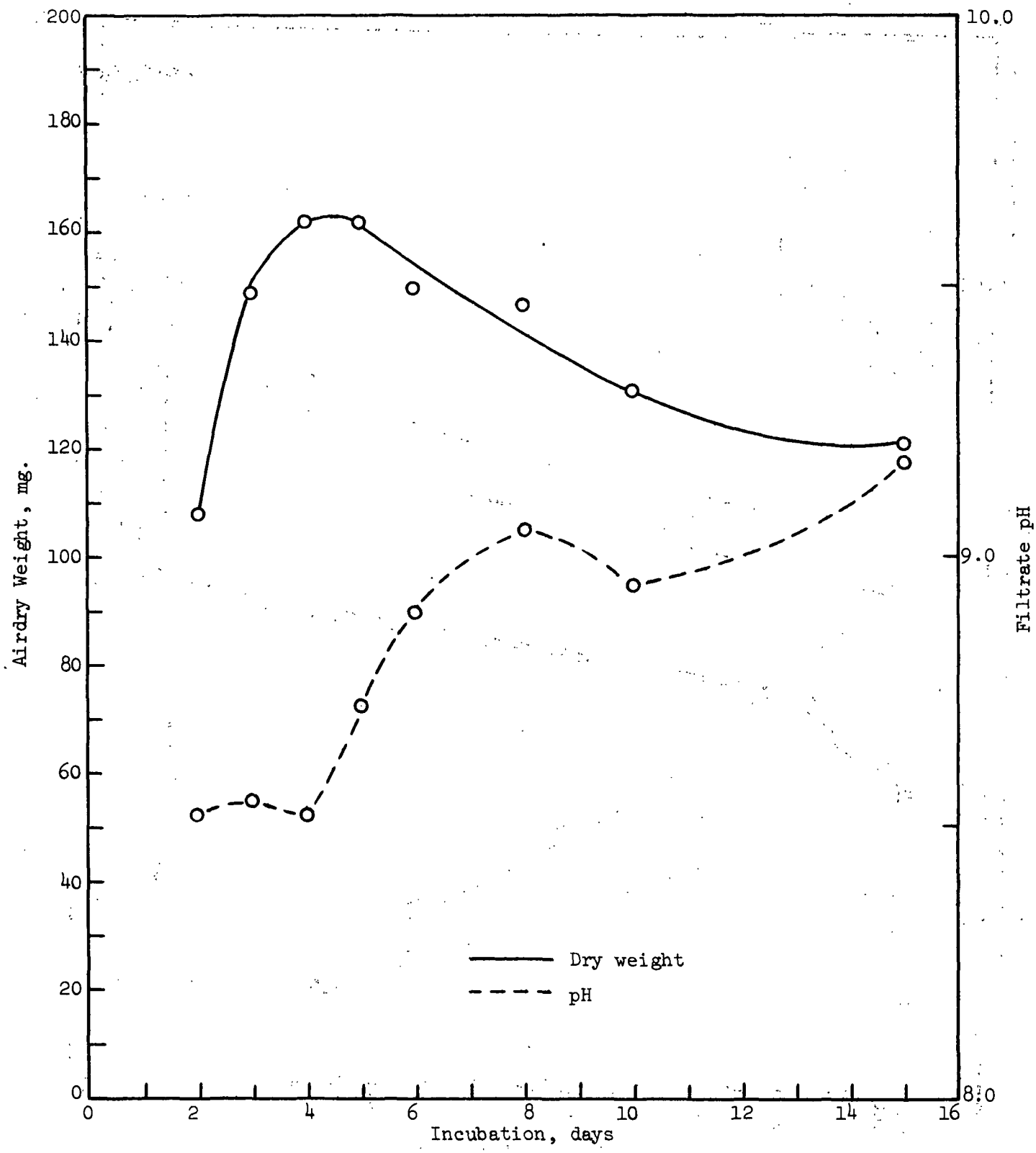

Figure 11. Partial Growth Curve for Fusarium Sp. on 50% NSSC Spent Liquor

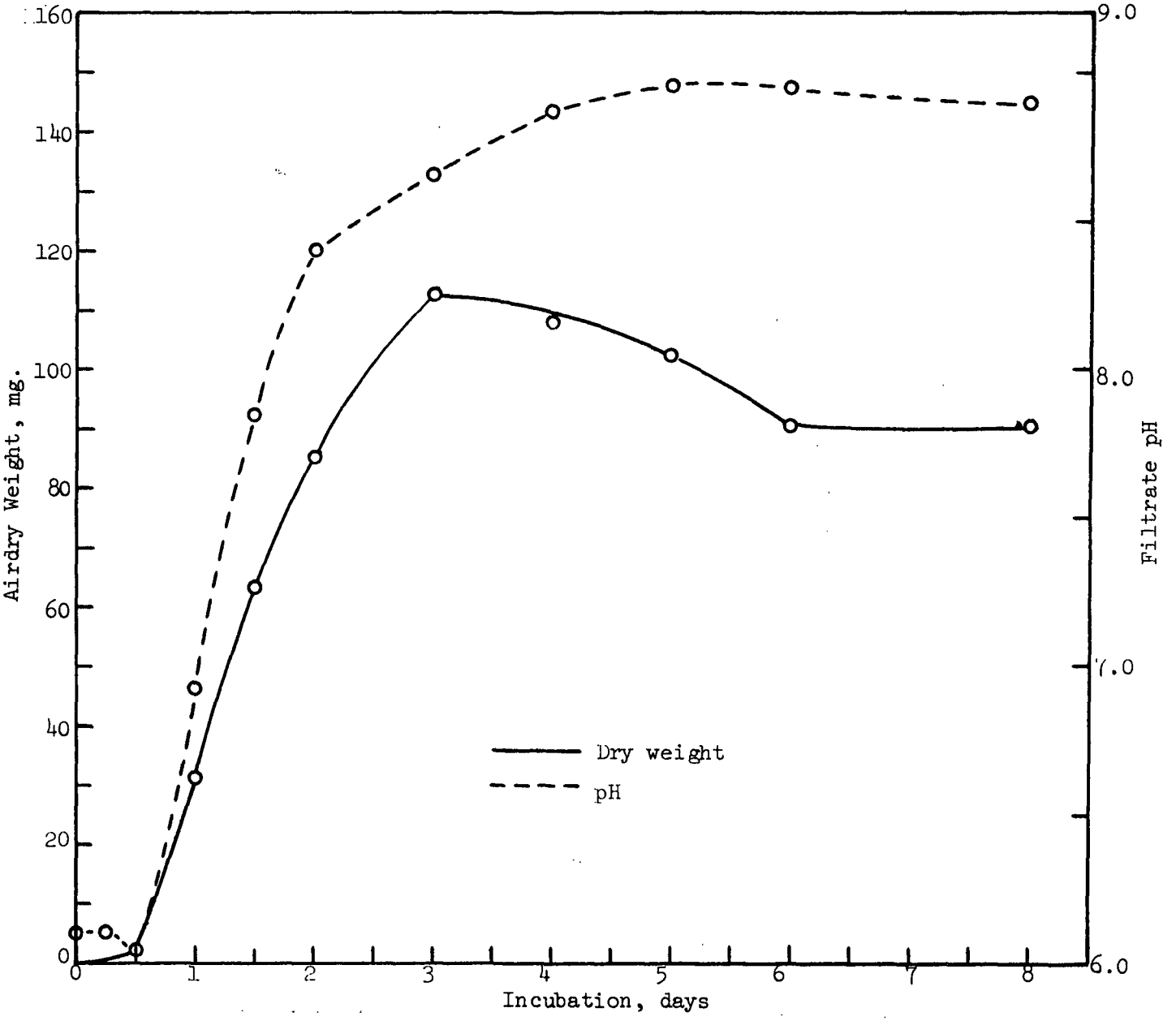

Figure 12. Growth Curve for M. rouxii on 25% NSSC Spent Liquor

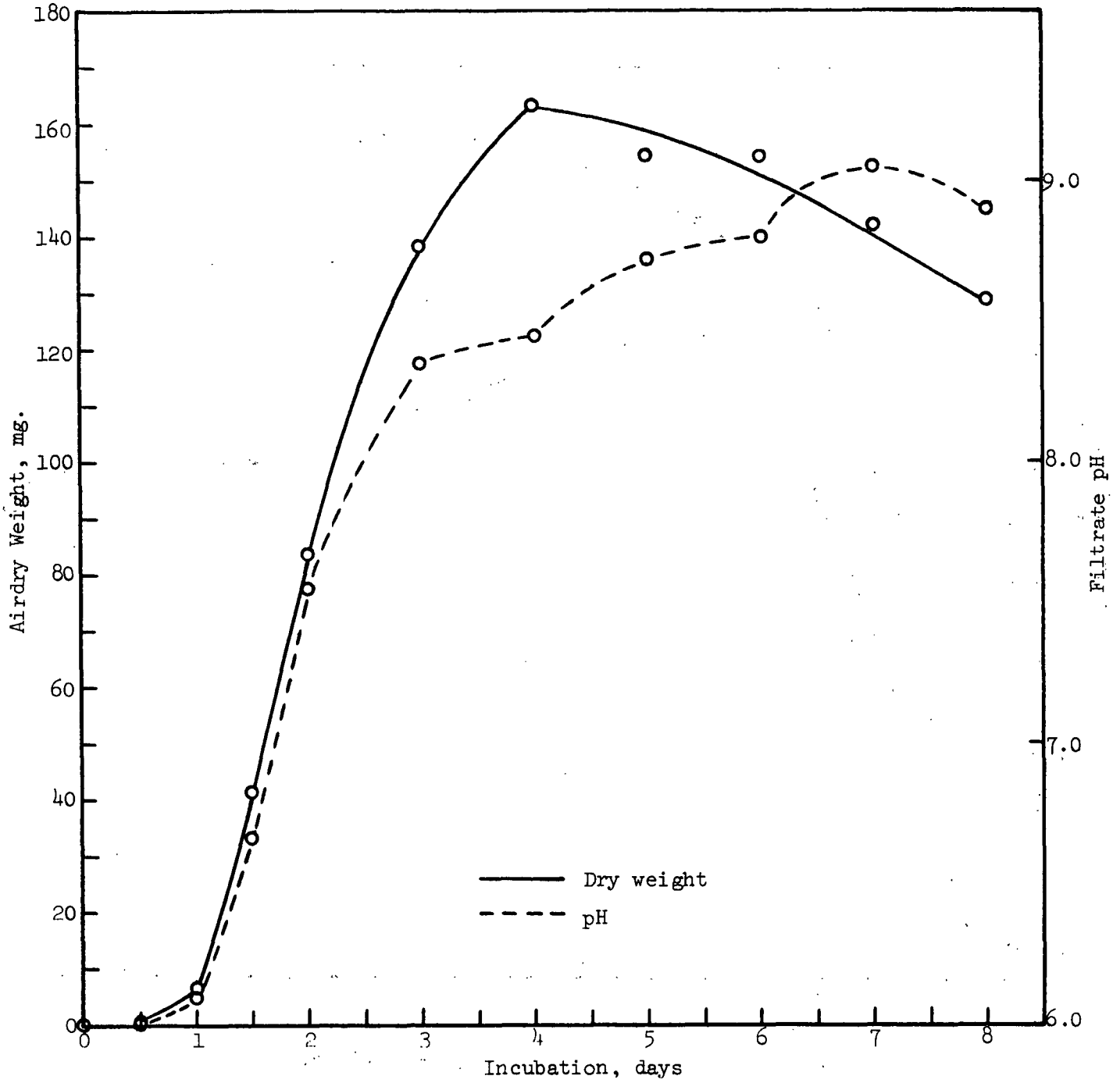

Figure 13. Growth Curve for Fusarium Sp. on 25% NSSC Spent Liquor

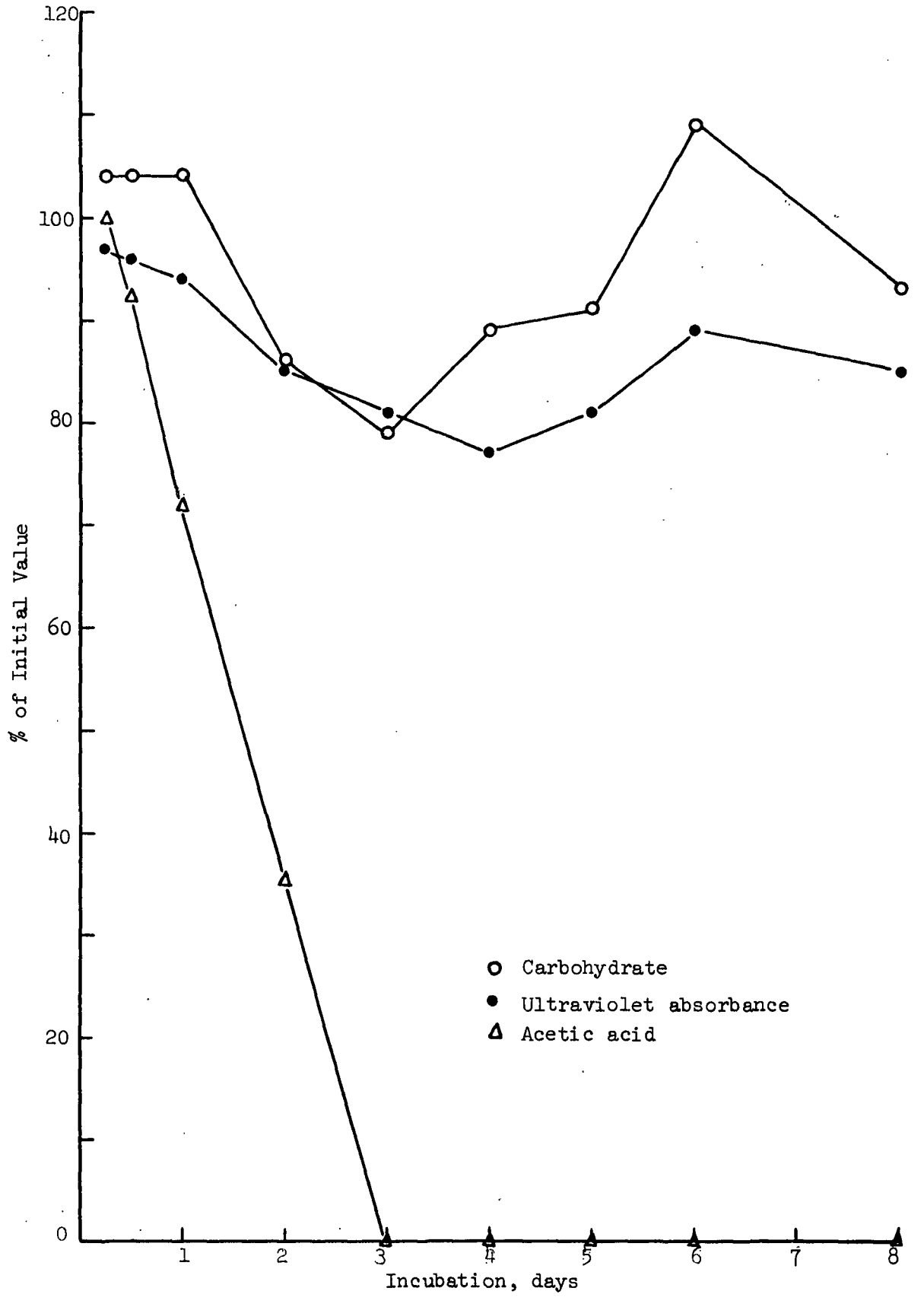

Figure 14. Utilization of Carbon Sources by M. rouxii

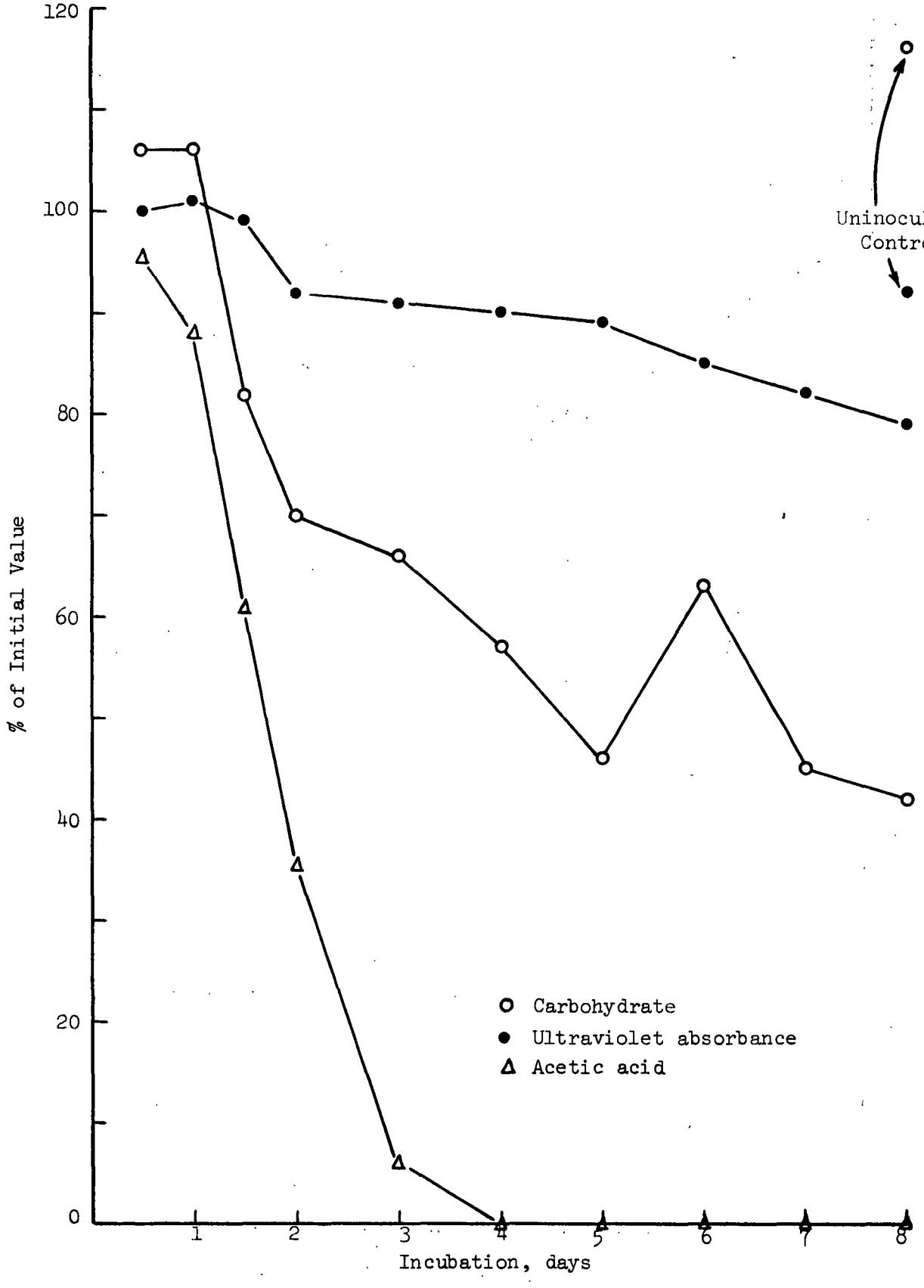

Figure 15. Utilization of Carbon Sources by Fusarium Sp.

DISCUSSION

PHASE I INVESTIGATIONS

Comments made in the first report about growth data such as found in Table I still apply, i.e., these data cannot be used to estimate maximum growth rates. Rhizidiomyces is not an organism with filamentous mycelia in the sense that the other studied fungi are. It is of interest only as a rare representative of the chitin-cellulose cell wall composition class. Unfortunately, its behavior in Tables II and III probably reflect more its morphology than its mycelial wall chemistry. The results for the other four organisms in Tables II and III and Fig. 3-7 must be regarded as somewhat tentative. Due to our difficulties in growing these organisms (see Table I) in large quantities, several batches had to be harvested, freeze-dried and stored until a sufficient quantity of mycelia was accumulated. One such batch apparently freeze-dried poorly and, when combined with the remaining mycelia, it led to poor (speckled) sheet formation. Handsheets that are well-formed at, for example, the 20% level must be made and compared before passing final judgment here.

It is evident from the results in Table IV that P. debaryanum mycelia incorporated into handsheets impart essentially identical changes in the strength characteristics of the handsheets regardless of the strength of the control pulp with which they are mixed. Apparently, the mycelial strands tended to bond among themselves to about the same degree independent of the presence of the aspen or spruce fibers. Taken together with the electron micrographs, such as shown in Report One, these data suggest a predominance of intramycelial bonding in the sheets, although some mycelia to fiber bonding does occur. Due to a substantial disparity in size, it would seem that some pretreatment of the wood pulp that

would give rise to greater fibrillation might lead to the formation of more mycelia to fiber bonds and improve the sheet. One member of our staff has suggested that these sheets suffer from a kind of "overbonding" which leads to the observed drastic losses in tearing strength and that it might be possible to pretreat the mycelia to prevent this.

While the use of a different control pulp has been the only effort made to modify the wood pulp component, two procedures used in our arbitrary workup of mycelia were examined, namely, freeze-drying and beating (British disintegrator) of the mycelia prior to handsheet formation (Tables V-VIII). S. ferax was chosen for this study since it performs well in sheets up to about the 25% level. The arbitrary 600 counts on the British disintegrator that was in routine use was found to be in a reasonable range; it may be possible to increase tensile strength slightly by further beating of the mycelia. Freeze-drying the S. ferax mycelia may have an adverse effect on sheet properties but it isn't very significant. Thus, our arbitrary processing of mycelia (steaming and washing procedures have not been investigated but are thought to be of little consequence) would seem to be satisfactory for this initial work since there appear to be only minor consequences detectable in the handsheet evaluation data.

It was necessary to confine most of our Phase II efforts to a few organisms, primarily Mucor and Fusarium. It may be recalled that organisms to be used in Phase II were to be the "promising" organisms of Phase I. The decision as to which organisms were promising was not a simple matter since the criteria for passing judgment had not been agreed upon. From the point of view of handsheet performance, S. ferax was the prime candidate; however, it was known that this organism required a complex nutrient medium in Phase I, and, on that basis, it lined up as the poorest candidate for Phase II work. Nevertheless, we tried

S. ferax initially but soon decided to test all of the organisms of Phase I since there were only five at the time. Mucor and Fusarium consistently demonstrated the greatest inclination to grow on the spent liquor and were selected for further study since they are also quite representative of extremes in the mycelial handsheet properties (see Report One), Mucor providing the more porous, weaker yet well-formed handsheet. Conflicting results have been obtained with other organisms, but these studies have been too limited to draw conclusions. In this connection, it is well to note that some weak positive responses were seen in the data of Tables XI and XII but not in Fig. 10; however, these three sets of data are not directly comparable because (1) the effects in Table XI are supported by the asparagine-glucose medium, (2) the effects in Table XII occurred with unsupplemented NSSC liquor, and (3) the effects in Fig. 10 were obtained at a constant level of $(NH_4)_2HPO_4$ supplementation. Since some organisms are more susceptible than others to ammonia toxicity, the level of $(NH_4)_2HPO_4$ used is a possible explanation for the failure of organisms other than Mucor and Fusarium to respond in the experiment of Fig. 10. More likely, factors in generally feeble growth are inhibition and an apparent failure of the other organisms to grow on acetate or pentoses (Table XVIII). Despite these results, it could be that these organisms contain inducible rather than constitutive enzymes for the utilization of these substrates; that would mean that it should be possible to train them to grow on acetate and pentoses provided no inhibitors were present.

Although we use clarified NSSC liquor in much of the Phase II work, it should be understood that there is really little chemical difference between this and the as-received liquor as can be appreciated by examination of Tables IX and X. There may be no reason to do this in situations other than our experimental work where dry weights are used as a growth measurement. Although metal cations do not

appear to be a serious problem (Table XIV), the NSSC spent liquor does not allow growth of any of the organisms studied if it is not diluted about 1:1. This suggests that there may be dissolved gases or organic compounds (lignin and tannin derivatives?) present which are inhibitory above certain concentrations.

Turning attention to Mucor and Fusarium which do grow on the liquor, it is found that about all that is required is to dilute the liquor and provide nitrogen and phosphorus supplementation (Fig. 9). When this is done the observed growth is equivalent to or better than on defined media (Table XVI). From Fig. 14 it is evident that Mucor derives most of its carbon from the catabolism of acetate while Fig. 15 suggests that the Fusarium does likewise but also makes more use of the available carbohydrate. In both cases all detectable acetate is removed from the liquor. At one point in the investigation we were concerned about the substantial pH changes taking place during the growth cycle and made some effort to control it by buffering (Fig. 8). The best yields occurred when the initial pH was 6 (lowest value run) and the final pH was about 8 (the highest pH attained in the experiment).

The finding that Mucor and Fusarium thrive on acetate suggests that the pH rise is due to the removal of acetate from the liquor and in that sense is desirable. It would be of interest to do some further investigation of this pH change. For example, what are the effects upon yield to buffer the system more strongly at pH 6.0 or to continuously or continually add a nonmetabolizable acid to keep the pH near 6.0? In contrast with the pH rise noted for growth on the liquor, both Mucor and Fusarium cultures become acidic when they are growing on sugar substrates (Tables XVI and XVIII). Consistent with this is a final pH of 5 when growth was conducted on a mixture of 20% NSSC liquor and 2% glucose. These

organisms must produce some acids during their growth on sugars; no attempt has been made to identify these products.

The growth curves for Mucor and Fusarium in 25% NSSC spent liquor are quite similar (Fig. 12 and 13), and the greater yield with Fusarium is in agreement with the observation that Fusarium uses substantial amounts of carbohydrate as well as acetate as carbon source. Note also that Fusarium gave essentially the same growth curve and yield (Fig. 11) when grown on 50% NSSC as when grown on 25% NSSC (Fig. 13). Figures 14 and 15 reveal that small amounts of ultraviolet absorbing materials are disappearing from media upon incubation. It is probable that at least some, if not all, of this disappearance may be attributed to adsorption on mycelial surfaces and/or effects on extinction due to the pH changes which occur. The latter is not too likely since pH was found to have little influence on zero time liquor spectra. It has been found on several occasions that mycelia which are white when grown on the defined asparagine-glucose medium are brown when grown on the NSSC liquor. If there is any real utilization of ultraviolet absorbing materials, it is thought to be more likely in the case of Fusarium where the general trend of all curves in Fig. 15 continues downward for eight days despite the fact that the growth curve peaked at about four days. In contrast, the carbohydrate and ultraviolet curves in Fig. 14 show reversal soon after the Mucor growth curve has peaked. A possible interpretation is that as the death phase sets in desorption occurs concurrently with general deterioration within the population. In the case of the carbohydrate it is conceivable that some mycelial degradation occurs at this time. It has also been noticed (Fig. 15) that uninoculated controls show slightly increased sugar values with time which may indicate that there are oligosaccharides present which undergo gradual hydrolysis; however, the indole analysis is run in strong acid which makes this interpretation rather

tenuous. The reproducibility of this somewhat trivial observation would need further examination prior to interpretation.

The utilization of mixed carbon sources (the NSSC liquor would fall in this category) by fungi can be a complicated business involving, in specific cases, phenonema like enzyme induction, enzyme regulation, organism mutation, inhibitory substances, etc. If one were to grow a mixed culture (should not rule out this possibility) on a mixed carbon source, the situation is compounded further, perhaps beyond our capacity to untangle it. Carbon-to-nitrogen ratios are known to be an important factor in yield data. Because of the mixed carbon source we are using and the fact that there is not uniform utilization of all carbon sources therein, it might be wise to extend the range of $(NH_4)_2HPO_4$ concentrations we have tried. Also, it would be desirable to compare ammonium to nitrate, nitrite, etc.

CURRENT STATUS AND OUTLOOK

The original objectives in this work were to grow and evaluate several fungi on defined media with regard to the properties of mycelial paper made from them (Phase I), and to examine the possibilities of growing promising fungi on NSSC spent liquor (Phase II). The funds for this research are depleted rather severely at this point, so it is well to take stock of the present situation.

In a limited sense the objectives have been met. In Phase I work, eleven organisms have been grown and their mycelial papers subjected to a standard paper evaluation regimen. We ourselves are not completely satisfied with the evaluation of the four organisms most recently acquired from the ATCC (see Discussion), but a repeat check on these results at the 20% mycelia level should serve to resolve any doubts. From the point of view that there are still many organisms that could be evaluated or that many other kinds of evaluation could be made, work has barely begun. To date it does not appear that mycelial papers are notable for their strength properties although some mycelia perform satisfactorily when incorporated at levels up to 25% (some results suggest a possible optimum at this level). It is pertinent to observe that in the patent description these papers are claimed to have desirable wet strength properties; no tests of this type have been made yet. Although proposed in the last report, tests other than those such as depicted in Table II have not been conducted. Neither have there been extensive attempts to modify either the mycelia or the control pulp prior to sheet formation. Since it has been possible to make at least 50% sheets from the mycelia of all fungi tested, investigations in any of these directions should be tenable if desired.

With a couple of possible exceptions it is somewhat doubtful that a fungus can be found which would give mycelial paper properties far outside the ranges already encountered. Chances for improvement are probably greater in the modification of mycelia or wood pulp in existing cases. Mycelial cell wall composition class as a basis for different mycelial sheet properties leaves much to be desired since, although there may be substantial differences between classes generally, substantial variation within classes has also been encountered. One could use this as grounds to conduct tests with more organisms selected at random within a given class. It is disconcerting to consider the possibility that some differences observed in paper evaluation data may reflect primarily differences in sheet formation, e.g., if it were possible to make Phytophthora sheets that were more uniform, would the evaluation picture change considerably?

Phase II research indicates that only a limited number of species will grow readily on the NSSC spent liquor, but that does not automatically rule out all of the others. Those that do grow well, Mucor and Fusarium, have growth rates on the order of 1 g./l./day (in Fig. 12 and 13 the rates are 0.9 and 1.1, respectively). As stated earlier, both of these organisms remove all detectable acetate. In addition, if harvested at the right time, Mucor removes about 20% and Fusarium about 40% of the total carbohydrate. On the other hand, the efficiency with which substrate is converted to mycelia is quite low (about 10%) in both cases, but this is thought to reflect the fact that acetate is the major carbon source utilized during log phase growth. Most of this substrate is probably converted to carbon dioxide rather than cell material due to its position in metabolic pathways. If one were to consider that the small amounts of carbohydrate utilized are the principal building blocks for mycelia synthesis, the efficiency picture would be reasonable (>30%). Efficiencies may be still higher if only a particular sugar

in the mixture is so utilized (not investigated). There is some indication of a lag period in carbohydrate uptake which may mean that even in these organisms induction is necessary for sugar utilization (primarily pentoses). There is also the possibility that greater reduction of B.O.D. might be realized by staging the mycelial growth either with the same or different organisms. Although those fungi other than Mucor and Fusarium have not been examined sufficiently, they might perform in a staged process; for example, it is conceivable that some of these would grow after the acetate had been removed by Mucor or Fusarium. In the interest of all growth studies, the reasons which underlie the necessity for NSSC liquor dilution deserve investigation.

The research to be conducted with the remaining funds will be chosen carefully with mileage in mind. Both Phase I and Phase II investigations are apt to be involved, but the acquisition of new organisms is not contemplated.

ACKNOWLEDGMENTS

Constructive commentary offered by Mr. J. Conkey, Drs. G. A. Nicholls, N. S. Thompson, and C. L. Garey in the course of this investigation is gratefully acknowledged. Also we are indebted to Dr. R. A. Parham for electron micrographs of mycelial paper.

LITERATURE CITED

1. Fuller, M. S. Personal communication, 1971.

2. Ashwell, G. Colorimetric analysis of sugars. In Methods in enzymology. Vol. III. p. 79. New York, Academic Press, 1957.

THE INSTITUTE OF PAPER CHEMISTRY

John A. Carlson,
Research Fellow

Morris A. Johnson
Research Fellow

Dean W. Einspahr
Senior Research Associate

John W. Swanson, Director
Division of Natural
Materials & Systems